STUDIES ON HUMAN ALPHA-1-PROTEINASE INHIBITOR

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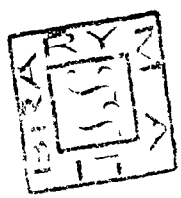

CERTIFICATE

I certify that the work presented in the following pages has been carried out by ***Ms. Kiran Misra*** *and that it is suitable for the award of M.Phil degree in Biotechnology of Aligarh Muslim University, Aligarh.*

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ABSTRACT

One of the proteinase inhibitors that has been studied in great detail is human alpha-1-proteinase inhibitor (alpha-1-PI). Alpha-1-PI is a single chain glycoprotein with a molecular weight of 51,000. Although it inhibits serine proteinases such as trypsin, chymotrypsin and elastase, its target enzyme appears to be neutrophil elastase.

The objective of this study is to investigate the kinetic stability of alpha-1-PI in the acidic and alkaline pH ranges. Alpha-1-PI was isolated from discarded human blood and subjected to 50-80% salt fractionation. It was further purified by DEAE-cellulose chromatography followed by chromatography on Con A Sepharose 4B column. The preparation was found to be homogeneous both with respect to charge and size as indicated by analytical gel chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing and non-reducing conditions.

The purified alpha-1-PI was found to be sensitive to pH at extreme values. At neutral pH, the inhibitor did not lose activity for 12 hours at 30°C. In acidic pH range below 5.0, precipitation of the inhibitor occurred. This precipitation was reversed by 8M urea without regain of inhibitory activity. Above pH 10.0 there was a measurable inactivation of inhibitor. At pH 11.0, the time course of inactivation was investigated at four temperatures namely 25°C, 30°C, 35°C and 40°C. The buffer used was 10mM sodium carbonate buffer,

pH 11.0 containing 0.15M NaCl. The inactivation process followed first order kinetics whence first order rate constant, k, was determined at four temperatures. From an Arrhenius plot of lnk versus 1/T, the activation energy for inactivation was computed to be 18.74 kcal/mole. As judged from the value of activation energy, alkaline inactivation of alpha-1-PI at pH 11.0 does not appear to be accompanied by major conformational changes in the protein. This is clear from our result on circular dichroic spectra of alpha-1-PI at pH 8.0 and pH 11.0 in far UV region.

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Kiran Misra

Aligarh **KIRAN MISRA**

14 December, 1995

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LIST OF ABBREVIATIONS

Alpha-1-PI	Alpha-1-proteinase inhibitor
BAPNA	Alpha-N-benzoyl-DLarginine-p-nitroanilide
$(NH_4)_2\ SO_4$	Ammonium sulphate
$CaCl_2$	Calcium chloride
Kcat	Catalytic rate constant
Con A	Concanvalin A
DEAE-cellulose	Diethyl amino ethyl cellulose
KD	Kilodalton
$MgSO_4$	Magnesium sulphate
$MnCl_2$	Manganese chloride
Km	Michaelis constant
Rm	Relative mobility
NaCl	Sodium chloride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tris	Tris (hydroxymethyl) amino methane

Introduction

INTRODUCTION

Human plasma contains a number of proteinase inhibitors which together represent 10% of the total plasma proteins (Travis & Salvesen, 1983). Protein proteinases are an important group of proteins capable of inhibiting the activity of proteolytic enzymes. They are ubiquitous and are found in all forms of life (Laskowski & Kato, 1980; Travis & Salvesen, 1983). Their primary function is to restrict and control the action of proteinases. These inhibitors act on four major classes of proteinases, viz., serine proteinases, cysteine proteinases, acid proteinases and metallo proteinases (Laskowski, 1986). Serine proteinase inhibitors (serpins) have been most extensively studied. Based upon amino acid sequence and disulphide bond topology, serpins have been classified into thirteen different families. They are listed in Table I. The three dimensional structure of inhibitors belonging to ten of the thirteen different classes has been determined either by X-ray crystallography or by 2D NMR spectroscopy (Bode, et al., 1985; Bode, et al., 1987; Huber & Carell, 1989).

Inhibitors of serine proteinases are known to be involved in phagocytosis, coagulation, complement activation and fibrinolysis. Quite often, the primary function of serpins is regulation of proteolytic events

TABLE I

FAMILIES OF PROTEIN INHIBITORS OF SERINE PROTEINASES

	Animals
1	Bovine pancreatic trypsin inhibitor (Kunitz) family[a]
2.	Pancreatic secretory trypsin inhibitor (Kazal) family[b]
3.	Ascaris inhibitor family[c].
4.	Serpin family (mechanistically distinct)[d]
5.	Hirudin family[a]
	Plants
6.	Soyabean trypsin inhibitor (Kunitz) family[e]
7.	Soyabean proteinase inhibitor (Bowman Birk) family[a]
8.	Potato 1 family[f]
9.	Potato 2 family[g]
10.	Barley trypsin inhibitor family[h]
11.	Squash inhibitor family[i]
	Microbial
12.	Streptomyces subtilisin inhibitor (SSI) family[a]
13.	Other families

a. Laskowski (1986).
b. Laskowski, et al. (1980).
c. Babin, et al. (1984).
d. Carrell and Travis, (1985).
e. Hejgaard, et al. (1983).
f. Graham, et al. (1985a).
g. Graham, et al. (1985b).
h. Odani, et al. (1983); Campas and Richardson (1983); Mahoney, et al (1984).
i. Wieczorek, et al. (1985); Joubert (1984).

associated with the number of biochemical pathways (see Fig. 1). The major examples of serpins are alpha-1-proteinase inhibitor (alpha-1-PI) or alpha-1-antitrypsin, alpha-1-antichymotrypsin, antithrombin III and alpha-antiplasmin. Human alpha-1-PI has been the subject of extensive investigations primarily because of its pathological importance (Erikson, 1965). The inhibitor is primarily synthesised in liver but it has been identified histochemically in monocytes and macrophages. Alpha-1-PI is primarily a defence protein whose function is to protect the tissues from attack by released proteolytic enzymes.

The alpha-1-PI gene comprises seven exons and six introns over 12.2 kb of chromosome 14 (Long, et al., 1984; Rabin, et al., 1986; Shen, et al., 1987) and is highly pleomorphic. As many as 75 alleles of the inhibitor have been identified so far. Besides normal alpha-1-PI alleles, there are deficient alleles that are associated with low serum levels of the inhibitor. The deficient alleles Z, S and $M_{procida}$ have been studied more extensively than the others (Travis & Salvesen, 1983; Curiel, et al., 1989; Takahashi, et al., 1988).

The inhibitor competitively inhibits the target enzyme which binds a substrate-like region in the inhibitor (Laskowski & Kato, 1980) in the molar ratio of 1:1. Most of the serine proteinase-directed inhibitors react with cognate enzymes according to a common, substrate-like standard mechanism (Laskowski & Kato,

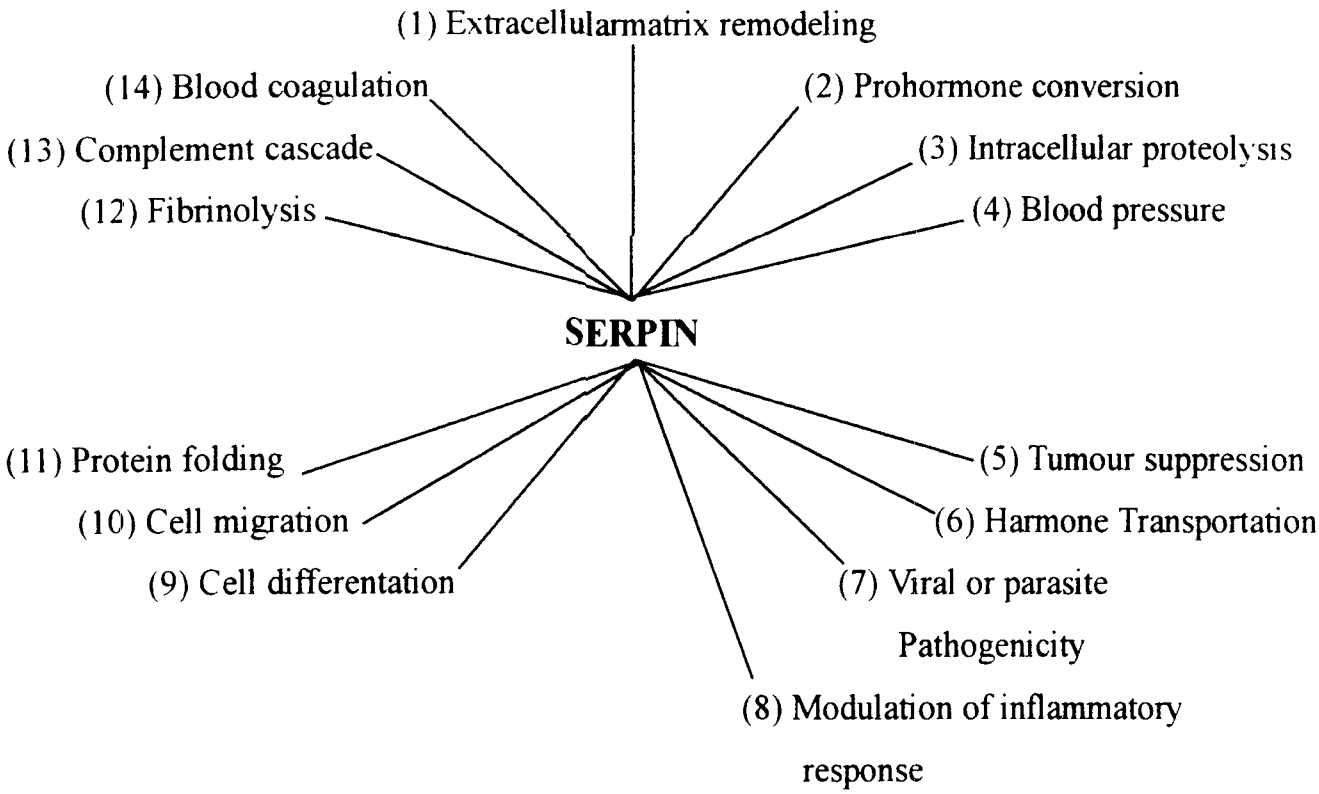


Fig.1 : Multiple regulatory functions of serpins
(Potempa. et al., 1994).

1980; Huber & Bode, 1978). The target enzyme binds to the inhibitor through the amino acid residue P1 at or near the active site region. The peptide bond between P_1 and P_1' known as reactive site peptide bond, is hydrolyzed during the complex formation between the inhibitor and enzyme with Kcat/Km of 10^4-10^6 M^{-1} sec^{-1} (Travis & Salvesen, 1983).

The rate constants for the association of various proteinases with human alpha-1-PI are listed in Table II (Travis, et al., 1985). As it is apparent from the values of association rate constants, the significant role of inhibitor is the regulation of proteinase activity of neutrophil elastase.

Primary structure of alpha-1-PI

Human alpha-1-PI is a protein consisting of single polypeptide chain containing 394 amino acid residues. The amino acid sequence was deduced both by protein sequencing methods (Carrell, et al., 1979; Carrell, et al., 1982) and by the cloning and sequencing of cDNA for the inhibitor from human and baboon. (Kurachi, et al., 1981). The amino acid sequence of alpha-1-PI is depicted in Fig. 2. Table III shows the amino acid composition of inhibitor as deduced from the sequence of amino acid residues. The number of acidic amino acid residues is 56(24 Asp and 33 Glu) which is greater than that of basic amino acid residues (54; 13 His., 34 lys

TABLE II

RATE OF ASSOCIATION OF PROTEINASES WITH HUMAN ALPHA-1-PROTEINASE INHIBITOR

S.No.	Enzymes	Rate constant[a] (M^{-1} sec^{-1})
1.	Neutrophil elastase	6.5×10^7
2.	Pancreatic elastase	1.0×10^5
3.	Cathepsin G	4.1×10^5
4.	Pancreatic trypsin	4.2×10^4
5.	Factor Xa	2.0×10^2
6.	Plasmin	1.9×10^2
7.	Thrombin	4.8×10^1

a. Travis, et al., (1985).

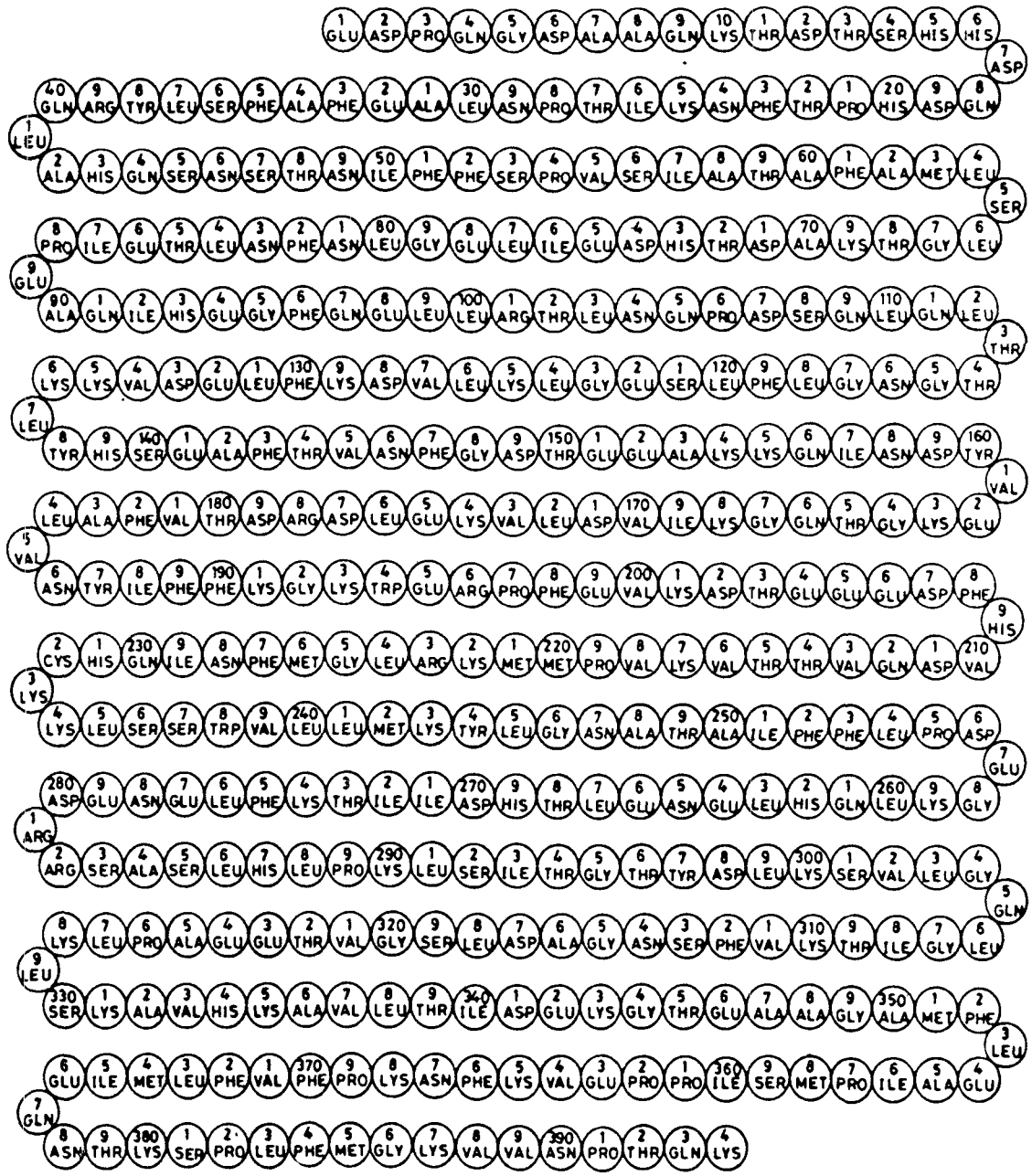


Fig.2: AMINO ACID SEQUENCE OF HUMAN ALPHA 1 PROTEINASE INHIBITOR

TABLE III

AMINO ACID COMPOSITION OF HUMAN ALPHA-1-PROTEINASE INHIBIITOR

S.No.	Amino acid	Number of residues[a]
1.	Phenylalanine	27
2.	Tyrosine	6
3.	Tryptophan	2
4.	Aspartic acid	24
5.	Glutamic acid	33
6.	Asparagine	19
7.	Glutamine	18
8.	Histidine	13
9.	Lysine	34
10.	Arginine	7
11.	Serine	21
12.	Threonine	31
13.	Methionine	9
14.	Cysteine	1
15.	Glycine	22
16.	Proline	17
17.	Alanine	24
18.	Valine	24
19.	Leucine	45
20.	Isoleucine	19
	Total	**394**

a. Deduced from amino acid sequence (Carrell, et al., 1982).

and 7 Arg) as a result of which the inhibitor is expected to possess a net negative charge at the physiological pH. The polypeptide chain consists of only one cysteine residue at position 232 which is flanked by His-231 and Lys-233 and is generally linked with glutathione or immunoglobulins (Laurell & Jeppson, 1975). In the native form the sulfydryl group exists in the reduced form which protects the inhibitor from inactivation by oxidants. The hydrophobic amino acids, namely alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan and methione, constitute 40% of the total amino acid residues in the inhibitor. Characterisation of various single amino acid substitutions at position 51 suggests that volume and flexibility of hydrophobic side chain at the site are critical for enhancing the stability of alpha-1-PI (Kwon, et al., 1994). It was reported that proline at position 391 as well as the nine amino acid-conserved sequence near the carboxyl terminal is critical for the efficient secretion of alpha-1-PI (Brodbeck & Brown, 1994).

The reactive site of alpha-1-PI is located near the C-terminal region around Met-358. The short segment of sequence around Met-358 is given in Fig. 3. There are mutants of alpha-1-PI where only the residue at P1 is replaced by Val (Valine mutant) or Arg (Pittsburg mutant) (Carrell & Travis, 1985) and the residues at positions P2, P1', P2', P3' and P4' remain the same as

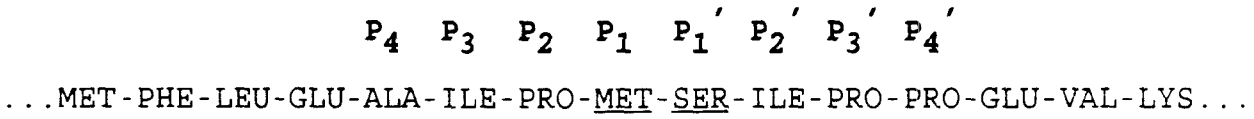


Fig. 3 : Amino acid sequence around active site residue Met-358 in human alpha-1-proteinase inhibitor.

in the normal alpha-1-PI. Substitution of Val-358 by similar hydrophobic amino acid residue, such as Ala, had no effect on the inhibitory activity of Valine mutant (Matheson, et al., 1986). However, Val mutant is more sensitive to heat inactivation than the normal alpha-1-PI (Travis, et al., 1985).

Physico chemical properties of alpha-1-PI

Alpha-1-PI is a single chain glycoprotein containing 11-13% carbohydrate. Its physico chemical properties are listed in Table IV. The molecular weight and the sedimentation coefficient $S_{20,W}$ values suggest a globular conformation of the inhibitor. The three oligosaccharide chains are linked to Asn-46, Asn-83 and Asn-247 (Travis & Salvesen, 1983; Carrell & Travis, 1985; Takahara & Sinohara, 1982). Interestingly the recombinant alpha-1-PI, devoid of carbohydrate, differs from the normal inhibitor in folding and refolding reactions. With the recombinant inhibitor, marked aggregation was observed during refolding reaction (Powell & Pain, 1992).

Structure of alpha-1-PI

Structure of alpha-1-PI modified at the reactive site peptide bond Met-358--Ser-359 has been analysed by Loebermann, et al., (1984) by X-ray diffraction at $3A^{O}$ resolution. The dimensions of the molecule are $67A^{O}$x $32A^{O}$ x $32A^{O}$. The polypeptide chain is arranged almost

TABLE IV

PHYSICO-CHEMICAL PROPERTIES OF HUMAN ALPHA-1-PROTENAISE INHIBITOR

S.No.	Property	Value
1.	Molecular Weight (KDa):-	
(i)	SDS-PAGE	49-52[a]
(ii)	Sedimentation equilibrium	49.7,[a] 54[b]
2.	Carbohydrate	13.4[a],11.2[b]
3.	No.of polypeptide chain	1[a]
4.	$E^{1\%}_{1cm}$ at 280 nm	5-5.3[a]
5.	Sedimentation coefficient, $S_{20,W}$	3.15[a], 3.65[b]
6.	Partial specific volume, $\bar{V}_2$, ml/gm.	0.725[a]

a. Travis & Salvesen (1983); Musiani & Tomasi, Jr.(1976); Pannell, et al., (1974).

b. Takahara & Sinohara (1982); Takahara & Sinohara (1983);

exclusively in well defined secondary structure elements that are documented in Table V and the stereoscopic drawing of the secondary structural elements of alpha-1-PI is shown in Fig. 4

The first 160 amino acid residues of the inhibitor in the N-terminal region predominantly form α-helix, whereas the residues in the C-terminal region containing reactive site peptide bond prefer to exist in β-structure. The inhibitor possesses a highly ordered structure with 40% β-sheet and 32% α-helix. Bruch, et al., (1988), obtained similar values for α-helix (35%) and β-structure (44%).

Evidently the dominant element is the large Sheet A consisting of six strands arranged antiparallely except strand 1 which is parallel to strand 2. Sheet B is also formed from six strands arranged antiparallely. Sheet B is approximately perpendicular to sheet A. Sheet C consists of 3 strands. Segment 202 to 223 forms a strongly twisted double stranded antiparallel ladder. Sheet A is relatively flat, Sheet B is strongly twisted, Sheet C has also a substantial twist. Parts of Sheet B and C are arranged to form a barrel located on one end of the elliptical molecule. The barrel is made of strands 1, 2 and 3 of Sheet C and strands 1, 2, 3 and 4 of Sheet B.

The helices are concentrated in the N-terminal part of the linear sequence. Helices A to E are in

TABLE V

SECONDARY STRUCTURAL ELEMENTS IN HUMAN ALPHA-1-PROTEINASE INHIBITOR[a]

helixes	sheets	turns	bulges
hA : 20-44 (kink at 28 Pro)	s6B : 49-53	thAs6B : 45-48	169-172
	s5B : 380-389	thBhC : 68-70	171-174
hB : 53-68	s4B : 369-378	thChD : 81-88 (1h: 81)	173-176
			(series of overlapping bulges)
hC : 69-81	s3B : 247-225		
hC1 : 83-87	s2B : 236-245	thDs2A : 105-110	
hD : 88-105	s1B : 228-233	ts2AhE : 122-127	
hE : 127-139	s6A : 290-299	thEs1A : 139-140 (1h: 139)	bs5B : 382-385
hF : 149-166	s5A : 326-342		
hF1 : 200-203 (one open turn)	s4A : 343-356	ts1AhF : 146-149	bs5A : 329-332
	s3A : 181-194	thFs3A : 166-181 (1h: 166) (series of bulges)	
hF2 : 232-236 (one open turn)	s2A : 109-121		
	s1A : 140-146		
hG : 259-264	s4C : 203-212	ts3AhF1 : 194-199	
hH : 268-278	s3C : 213-226	ts4Cs3C : 211-214	
hI : 299-306	s2C : 283-289	ts3Cs1B : 226-228	
hI1 : 309-312 (one open turn)	s1C : 362-367	ts1Bs2B : 233-236 (1h: 236)	
hI2 : 376-380 (one open turn)		ts2Bs3B : 244-248	
		ts3BhG : 256-259	
hI3 : 390-393 (one open turn)		thHs2C : 278-283	
		th11s5A : 318-325	
		ts5As4A : 341-344	
		ts4Bs5B : 377-380 (1h: 380)	
		ts5Bc-ter : 389-394	

Residues at the termini of helixes are included if at least one of their main-chain conformational angles is canonical; stands of sheets are defined similarly, appropriate hydrogen bonds are not always made by these residurs hX, helix X, sXY, strand X in sheet Y; thXh Y, turn between helix X and helix Y; bsXY, bulge in strand X of sheet Y, 1h, left-handed helical conformation

(a). Huber and Carrell (1989)

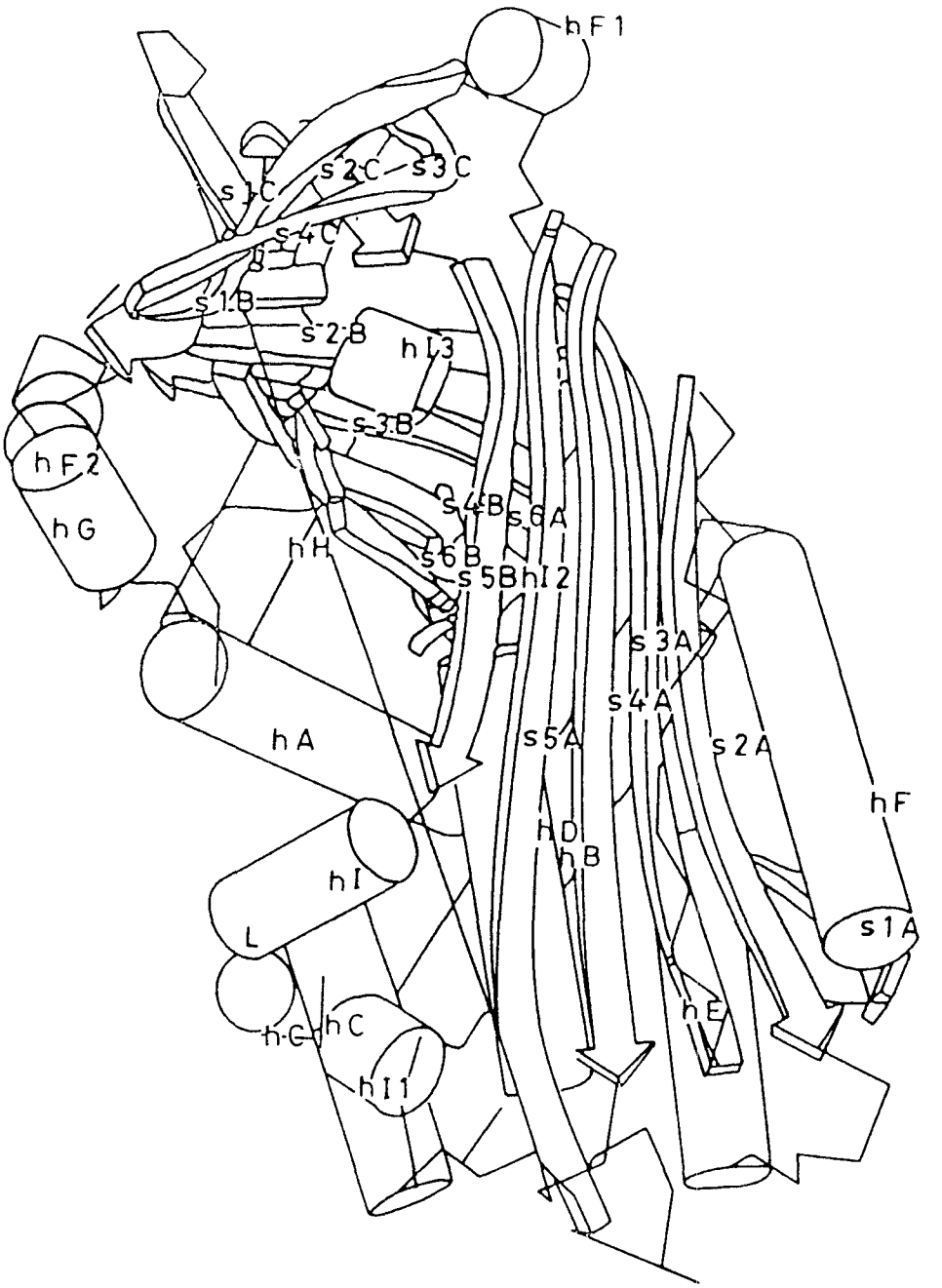


Fig.4: Poly peptide chain folding of human alpha-1-proteinase inhibitor. (Huber & Carrell, 1989).

Secondary structure elements are represented by arrows 'Sheet Strands and cylinders (helixes) and marked according to Table V. Residue numbers refer to alpha-1-PI and the alignment in Table V. Residues C-terminal to 358 have 100 added to their number in the plot.

close proximity on one side of Sheet A. Residues 21 to 147 may be regarded as a helix rich domain built from helix A, strand 6 of Sheet B, helices B to D, strand 2 of Sheet A and helix E. Helix B is internal and characterised in the linear sequence by a long segment of apolar residues. Helix F leans on the opposite side of Sheet A, helices G and H are associated with the β-barrel.

Sheets 2A, 3A, 2B, 3B, 4B, 5B and 6B are buried and characterized by linear segments of apolar residues. Sheet B is largely buried in the interior of the molecule between Sheet A and helix rich domain. The charged residues form a shell around the hydrophobic core, though the distribution is not uniform. Lys and Arg are concentrated on the 'barrel' end of the ellipsoidal molecule while the acidic residues are more abundant on the opposite end.

In the nicked species the new N and C termini conform to this arrangement : Ser-359 is located on the 'barrel' side and Met-358 is on the opposite end. The molecule has substantial dipole moment. The conversion of virgin inhibitor to nicked species involves insertion or removal of strand 4A.

The three carbohydrate attachment sites at Asn-46, Asn-83 and Asn-247 are all in bends of the polypeptide chain protruding from the surface of the molecule. Asn-

46 is located in a segment between helix A and strand 6B. Asn-83 is in the segment linking helices C and D, and Asn 247 is in the bend connecting 2B and 3B.

The structure proposed for native inhibitor has the susceptible peptide bond near the barrel end of the molecule. Strand 1B, a part of the barrel end, has the unique Cys-323 which is blocked presumably by cysteine or glutathione. Predicted (Garnier, et al., 1978) and observed secondary structures show a moderate correlation. In particular, strongly predicted helical regions agree with the observation. A remarkable exception is the region 325 to 355, which is strongly predicted as a helical region, but forms the central strand of Sheet A in the observed structure. Crystal structure studies have shown that cleaved and intact serpins differ essentially in the topology of Sheet A. This is five stranded in the intact molecules and six stranded after cleavage by insertion of strand 4A whose terminus has become free (Loebermann, et al., 1984; Wright & Huber, 1990).

In the intact native inhibitor the sequence Ala-348-Ser-359 is plucked out to form strained loop whereas in the nicked inhibitor Met-358 is separated from Ser-359 by a distance of 69A^{O} (Loebermann, et al., 1984). Both the forms, i.e., strained (S) and relaxed (R) conformation, differ in thermal stability (Carrell & Owen, 1985), circular dichroism spectra (Bruch, et al.,

1985), fluorescence spectral measurements (Bruch, et al., 1985) and antigencity (Agostini & Carrell, 1985; Zhu & Chan, 1987).

Reactive site loop (RSL) is an extended rigid loop that is complimentary to the substrate binding sites of a target proteinase. The RSL structure of the different classes of inhibitor is highly conserved despite the fact that the proteins themselves vary widely in overall structure (Laskowski & Kato, 1980; Read & James, 1986; Bode, et al., 1986; Hubbard, et al., 1991).

The proposed structure of the RSL of virgin human alpha-1-PI is given in Fig. 5. It is because of the flexibility and movement of some of the upstream RSL residues in and out of Sheet A that the serpin RSL can be thought as a dynamic rather than a static structure (Mast, et al., 1992).

While insertion of RSL is not essential for protease binding, it is a necessary second step required for inhibitor function. The presence of a charged residue at P14 can retard this insertion resulting in conversion of the serpin to a substrate (Lawrence, et al., 1994). A proposed model of serpin function suggests that the inhibitors have mobile RSL that can partially be inserted into Sheet A (Carrell, et al., 1991; Carrell & Evans, 1992). However, further insertion yields a latent inhibitor that is no longer reactive with its target protease but has increased thermal stability (Mottonen, et al.,1992; Lomas, et al.,

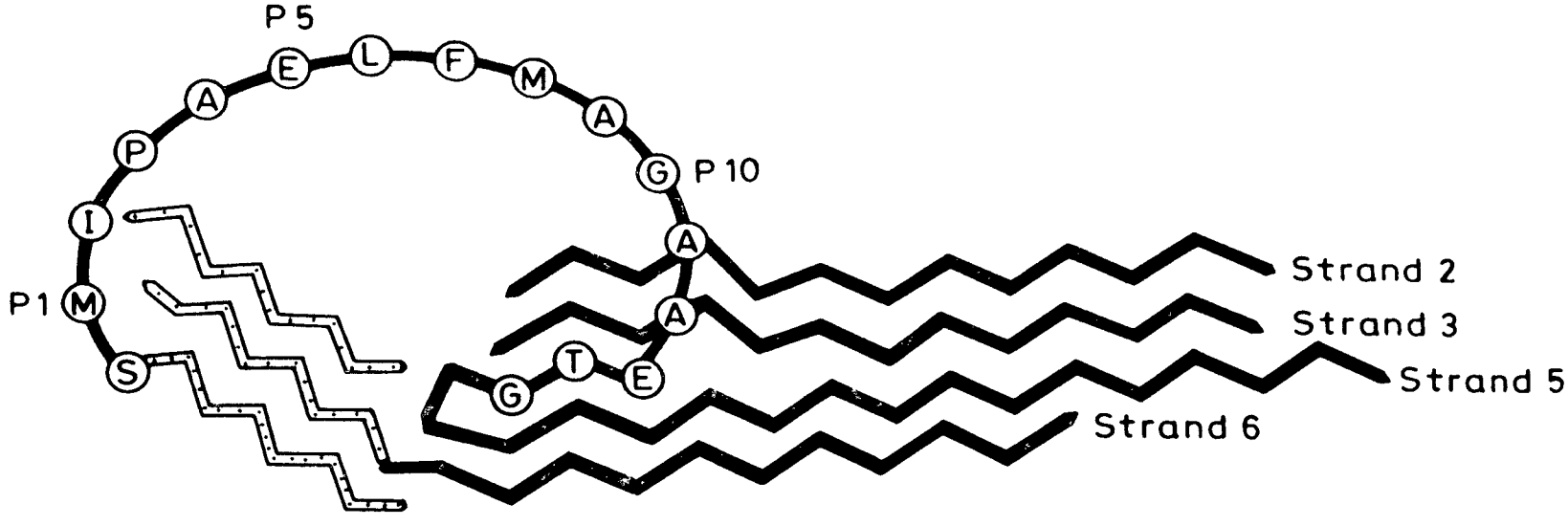


Fig. 5 : Proposed conformation of the RSL of virgin human alpha-1-proteinase inhibitor (Mast, et al., 1992).

1995). Blocking of loop insertion with synthetic loop peptides or by mutations within the loop converts the inhibitor into substrate (Carrell, et al.,1991; Schulze et al., 1990; Hood, et al., 1994).

Mutations within the region P9-P15 of RSL result in loss of inhibitory activity (Huber & Carrell, 1989). However, mutations in this region can lead to polymer formation through insertion of the loop of one molecule into the B Sheet of another (Lomas, et al., 1993; Mast, et al., 1992). This loop sheet polymerisation has provided an excellent explanation for the aggregation of the Z variant of human alpha-1-PI (Glu-342 to lys-342) in liver endoplasmic recticulum (Lomas, et al., 1992; Carrell, et al., 1994).

Despite the similarity of sequence, susceptibility to protease action, and presence of an elastase specific alanine at P1 position in the protease susceptible segment, ovalbumin is not an inhibitor of elastase but rather a good substrate for this enzyme (Wright, 1985). The reason for this was accounted for when the structure of plakalblumin (cleaved ovalbumin) and subsequently ovalbumin was shown (Stein, et al., 1990) which provided a model for uncleaved serpin (Wright, et al., 1990; Engh, et al., 1990) with 5 stranded pleated Sheet A bridged to -Sheet C by active site loop. All inhibitory serpins have a small residue (Thr, Ser, Ala or Val) at position 345 in strand 4A which is buried in

the cleaved species. Ovalbumin and its proteolytically modified form, plakalbumin, have an arginine in position 345 which for stereochemical reasons cannot be internal. This was suggested as the main reason for the lack of conformation transition upon insertion of strand 4A into Sheet A (Wright, et al., 1990; Schulze, et al., 1990). The recently characterised recombinant alpha-1-PI, Thr-345 --> Arg, Met-358 --> Arg variant (Schulze, et al., 1991) is not an inhibitor and is similar to ovalbumin concerning denaturation, stability, demonstrating the key role of residue 345 for both inhibitory activity and rearrangement of Sheet A.

Partial insertion of strand 4A is necessary for inhibitory conformation of the binding loop. A bulky residue at position 345 prevents this and renders the inhibitor into a substrate. The loop adopts an α -helical conformation in intact ovalbumin (Stein, et al., 1990), not competent for proteinase binding (Bode & Huber, 1991). It has been suggested also that active serpin inhibitors have an -helical loop which uncoils prior to or during proteinase attachment accompanied by insertion of strand 4A into Sheet A up to residue 349 (Skriver, et al., 1991).

Residues and interactions involved in complex formation between alpha-1-PI and serine proteinases

The interaction between alpha-1-PI and serine proteinases is very strong. The serine residue of the

proteinases contributes substantially towards the interaction. The inhibitor-enzyme complex is further stabilized by ionic and hydrophobic interactions. Thus the increase in temperature and ionic strength greatly enhance the inhibitor and enzyme complex (Saklatvala, et al., 1976; Satoh, et al., 1979). Moreover, the complex formation is found to be blocked by 20% dioxane (Cohen, 1975). The inhibitor on its reaction with maleic anhydride (Heimberger, et al., 1971), acetic anhydride (Fretz & Gan, 1978) or citraconic anhydride (Johnson & Travis, 1975), all of which react with lysyl residues in proteins, inactivated the inhibitor towards trypsin, chymotrypsin and elastase suggesting the involvement of lysine and arginine residues of alpha-1-PI in the complex formation between the inhibitor and its cognate enzyme. However, chemical modification of lysyl residues in proteins that alters the charge on the protein molecule or introduces a bulky group, is known to disrupt protein conformation (Ansari, et al., 1975, Qasim & Salahuddin, 1978). Accordingly, when less bulky group was introduced at the lysyl residues of alpha-1-PI without affecting its net charge, this chemical modification did not result in inactivation of the inhibitor (Busby, et al., 1977), and excluded the possibility of involvement of lysine residue of the inhibitor in complex formation. It has also been suggested that arginine (Cohen, 1973) and tyrosine (Feste & Gan, 1981) residues of alpha-1-PI might be

involved in its inhibitory activity.

Role of methionine residues in the inhibitory activity of alpha-1-PI

There are several lines of evidence which suggest the involvement of methionine residues of the inhibitor in its inhibitory activity (Janoff & Carp, 1977; Johnson & Travis, 1978, 1979; Carp & Janoff, 1979; Ohlsson, et al, 1980; Abrams, et al., 1981a). Oxidation of methionine residue, at positions P1 and P8 and also other few methionine residues, to methionine sulphoxide by the release of oxidants by neutrophil elastase during phagocytosis lead to the inactivation of the alpha-1-PI (Carp & Janoff, 1979). Substantial loss in the inhibitory activity of alpha-1-PI towards neutrophil and pancreatic elastases by unfractionated cigarette smoke (Janoff & Carp, 1977; Ohlsson, et al., 1980; Abrams, et al., 1981a) was prevented by antioxidants.

The inhibitory activity of the oxidised alpha-1-PI can be restored by methionine sulphoxide reductase (Abrams, et al., 1981b). This enzyme is likely to play a role in preventing unwanted tissue proteolysis in lung. Oxidation of methionine to methionine sulphoxide causes reduction in the interaction of alpha-1-PI with target enzyme presumably because of steric hindrance offered by the relatively bulky methionine sulphoxide at the reactive site (Loebermann, et al., 1984). If the

methionine at 358 position in the alpha-1-PI is mutated to valine then it was found that the Val mutant of alpha-1-PI was not inactivated upon oxidation as compared to the normal alpha-1-PI which did not react with pancreatic elastase upon oxidation (Rosenberg, et al., 1984).

Nature has provided such a vulnerable reactive centre for alpha-1-PI as to effect the ability to switch off alpha-1-PI which provides a means by which essential tissue breakdown can occur in the immediate vicinity of an inflammatory focus (Carrell, et al., 1982) and perhaps in the area of regrowth.

Biological significance of alpha-1-PI

Abnormal phenotypes of alpha-1-PI have been associated with a number of diseases including lung diseases, like eymphysema, (Lieberman, 1976), bronchietasis (Shin & Hn, 1993), pneumonia (Braun, et al., 1994), liver diseases like obstructive jaundice, fatal juvenile cirrhosis (Propst, et al., 1994), rheumatoid arthiritis (Zhang, et al., 1993; Chidwick, et al., 1994) and hepatocellular carcinoma (Scott, 1994). Alpha-1-PI has been reported to be involved in the pathogenesis of the lesions of Alzheimer's disease (Gollin, et al., 1992). Alpha-1-PI phenotypes of M1, M2, M1M2, M2M3, M1M3 were found in patients with Behcet's disease (Fakuda, 1992).

Alpha-1-PI can be proteolytically inactivated by several matrix metalloproteinases including interstitial collagenase, stromelysin, gelatinase and matrilysin (Ottonello & Dapino, 1993; Zhang, et al., 1994; Sires, et al., 1994). Reactive oxygen species in cigarette smoke (Travis & Salvesen, 1983) and coal dust (Huang & Laurent, 1993) leads to inactivation of alpha-1-PI. It has been reported that glutathione has protective role in alpha-1-PI inactivation by myeloperoxidase system of activated phagocytic cells in case of emphysema (Gressier, et al., 1994).

A review of literature mentioned above demonstrates an emphasis on the inactivation of alpha-1-PI primarily by oxidative modification of the critical methionine residue in the inhibitor. The inhibitor is known to undergo inactivation at extreme pH values in the acidic and alkaline range. In this dissertation we have focused our attention primarily on the alkaline inactivation of alpha-1-PI. Our results have shown that the inhibitor was fairly stable in the pH range 5-10, beyond which increase in pH caused irreversible inactivation of alpha-1-PI.

Experimental

EXPERIMENTAL

A. MATERIALS

1. Proteins

Bovine serum albumin (Lot no 100F-0249), ovalbumin (Lot no. 105C-8022), chymotrypsinogen A (Lot no.40F-8050), cytochrome c (Lot no. 09C-0088), myoglobin (Lot no. 85C-0138), bovine trypsin type III (Lot no.128F-0397) were purchased from Sigma Chemical Company, St. Louis, MO., USA.

2. Substrate for assay of proteolytic enzyme

Alpha-N-benzoyl-DL-arginine-p-nitro-anilide (BAPNA) (Lot no. 37F-0833) which was used in the assay of trypsin was obtained from Sigma Chemical Company, St. Louis, MO., USA.

3. Column chromatographic media

Diethylaminoethyl cellulose (DEAE-cellulose) (lot no. F/8/6011) was obtained from Sisco.Res.Lab. Bombay, India. Sephadex G-100, Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Con A Sepharose 4B (Lot no. 90C-0495) was obtained from Sigma Chemical Company, St. Louis, MO., USA.

4. Reagents used for gel electrophoresis

Reagents used in eletrophoresis with their sources in parantheses were : acrylamide (E. Merck, Dramstadt, Germany),N-N'-methylene bisacrylamide (Reanal, Budapest,

Hungary), N,N,N',N'-tetramethylethylenediamine (Ferak, Berlin, Germany), 2-mercaptoethanol (BDH,Poole, England), ammonium persulphate (E. Merck, Dramstadt, Germany), sodium dodecyl sulphate (BDH, Bombay, India), coomassie brillant blue R-250 (Sigma Chemical Company, St. Louis, MO., USA), and di-chlorodimethyl silane (BDH, Poole, England). Glycerol was obtained from BDH, Bombay, India.

5. Miscellaneous reagents

Methanol, acetic acid, glucose and chloroform were purchased from BDH, Bombay, India. Tris (hydroxymethyl) amino methane was obtained from Sisco, Res. Lab., Bombay, India. Sodium azide was obtained from Fluka, Switzerland and dimethyl sulphoxide was purchased from BDH, Poole, England. The rest of the chemicals used were of analytical grade. Glass distilled water was used throughout these studies.

B. METHODS

1. Measurement of pH

Elico digital pH meter model L1-122, was used for pH measurements in conjunction with Elico combined electrode at room temperature. The pH meter was standardized with 0.05M potassium hydrogen pthalate buffer, pH 4.0 in the acidic range and with 0.01M sodium tetraborate buffer, pH 9.2 in the basic range.

2. Optical measurements

Absorbance in the visible range was measured on a AIMIL photochem-8 colorimeter. In the ultraviolet region, light absorption measurements were performed on Cecil UV-double beam spectrophotometer, model CE-594 using silica cells of 1 cm path length.

The CD spectra were measured on a Jasco Spectropolarimeter model J-720 instrument, using a Sekonic X-Y plotter (model SPL-430A) and PC-A T286 compatible computer with 1024 Kb Ram. The pathlength of cell used was 0.1 cm.

The mean residue ellipticity $(\Theta)_\lambda$,

$$(\Theta)_\lambda = \frac{\theta_\lambda}{10 \times n \times C_p \times L}$$

where

θ_λ = ellipticity in mdeg at a given wavelength,

n = number of amino acid residues in the protein,

C_p = molar concentration of the protein, and

L = path length of the cell in cm.

3. Determination of protein concentration

Protein concentration was determined either by the method of Lowry, et al. (1951) or by spectrophotometric method.

(a) Lowry's method

To one ml of protein solution was added 5 ml of

copper reagant and after mixing the solution, it was allowed to stand for 10 minutes at room temperature. Then 1 ml of Folin-phenol reagant was added to the mixture and the contents were mixed well. The mixture was incubated for 30 minutes and the colour intensity was read at 700 nm. A calibration curve between optical density at 700 nm and protein concentration in milligram was obtained by the method of least squares (Fig. 6), using bovine serum albumin as standard. The linear curve was found to fit the equation :

$$(\text{Absorbance})_{700nm} = 1.67\ (\text{mg, protein}) + 0.009 \qquad (1)$$

(b) Spectrophotometric method

In a few experiments, the protein concentration was determined by measuring the optical density of protein solution in 0.1 M Tris-HCl buffer, pH 8.0 containing 0.15M NaCl, 1mM $CaCl_2$, $MnCl_2$ and $MgSO_4$ using appropriate values of specific extinction coefficient, $E^{1\%}_{1cm}$ at 280 nm. The values of $E^{1\%}_{1cm}$ used were : trypsin, 14.4 (Walsh, 1970) and alpha-1-PI, 5.3 (Pannell & Travis, 1974).

4. Chromatography

(a) Ion exchange chromatography

Alpha-1-PI was purified by DEAE-cellulose chromatography. In order to regenerate the resin, it was treated with one litre of 0.1 M sodium hydroxide and

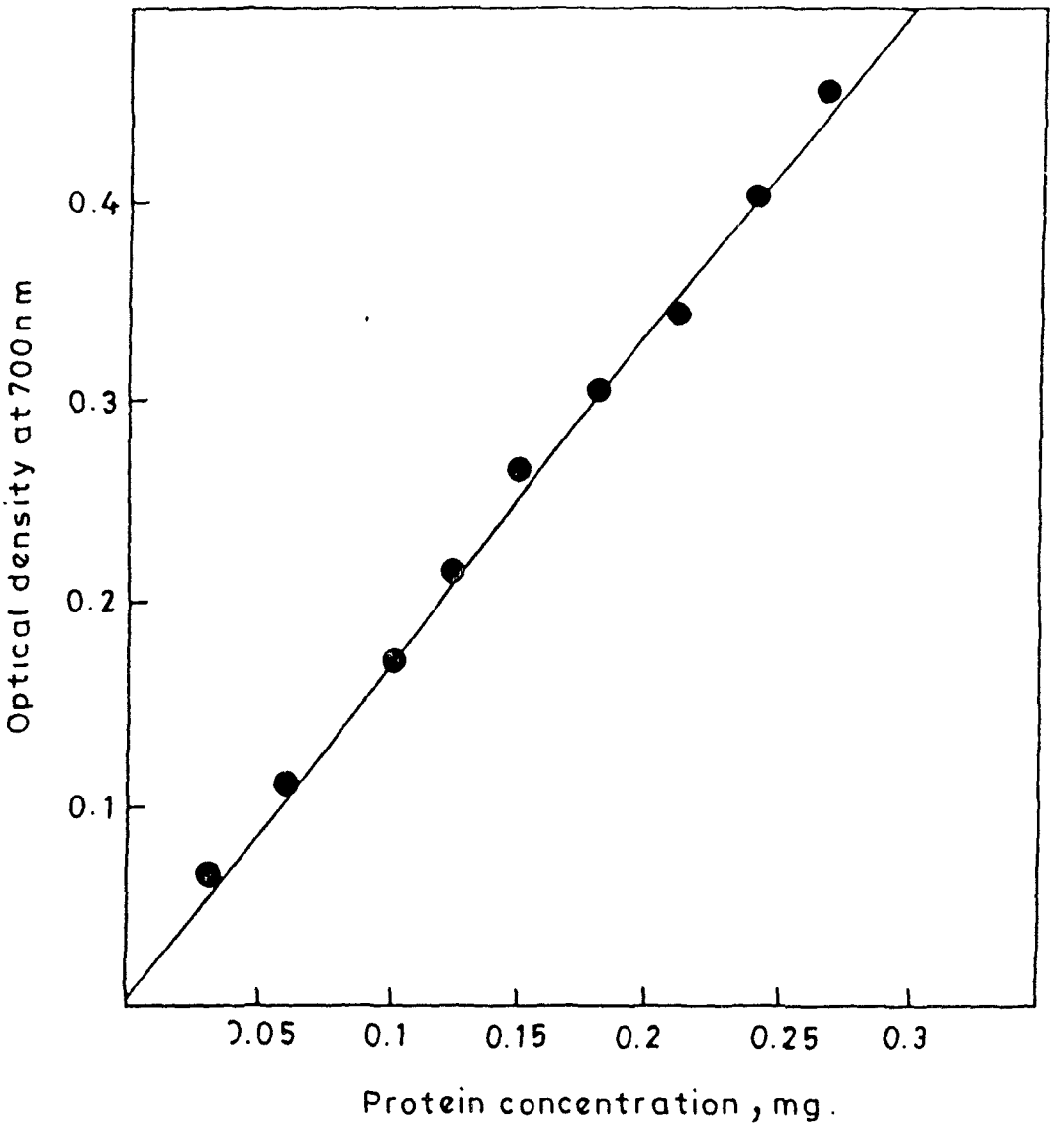


Fig. 6 : Calibration curve for the estimation of protein concentration by the method of Lowry, et al., (1951). Bovine serum albumin was used as the standard protein. The straight line drawn by the method of least squares follows the equation:

$$(\text{Absorbance})_{700} = 1.67\ (\text{mg, protein}) + 0.009$$

then washed extensively with distilled water till pH of the washing became neutral. After this, the ion exchanger was treated with one litre of 0.1 M hydrochloric acid and washed extensively with water till pH of the washing became neutral. The exchanger was packed into a column (2.5 X 5.8 cm) and subsequently equilibrated with 10 mM sodium phosphate buffer, pH 7.6.

(b) Affinity Chromatography

Con A Sepharose 4B gel was packed into a glass column (2.2 X 5.3 cm) and was subsequently equilibrated with the operating 10 mM Tris-HCl buffer pH 7.6, containing 0.15 M NaCl, 1mM $CaCl_2$, $MnCl_2$ and $MgSo_4$. The inhibitor was further purified on this affinity matrix.

(C) Sephadex gel chromatography

The alpha-1-PI was isolated on a Sephadex G-100 column (2.4 X 79 cm) which was packed according to the method of Ansari and Salahuddin (1973). The homogeneity of packing was checked by passing a band of 0.02% (w/v) solution of Blue Dextran through the column. The pattern of the elution profile showed uniform packing of the column. The elution volume (Ve) of Blue Dextran yielded the void volume (Vo) of the column. As can be seen in Fig.7 the dye eluted from the column as a symmetrical peak with an elution volume of 135 ml. The total volume of the column, V_t, was determined to be 358 ml. The column was equilibrated with 10mM sodium

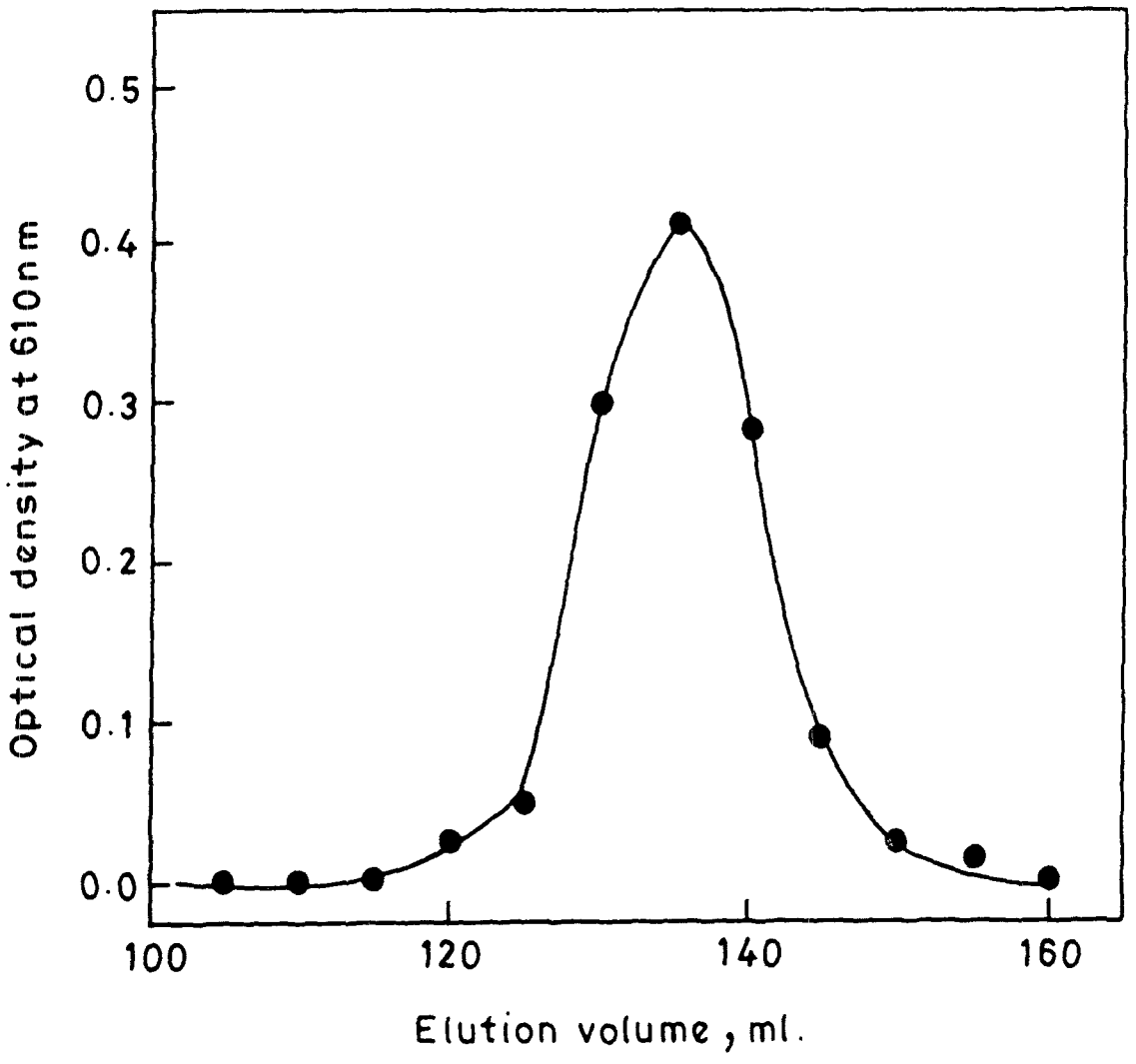


Fig. 7: Gel chromatography of Blue Dextran on Sephadex G-100 column.

About 10 mg of the sample was applied to the column (2.4 x 79 cm) equilibrated with 10 mM sodium phosphate buffer, pH 706 and eluted in 5 ml fractions at a flow rate of 20 ml/hr. The colour intensity was read at 610 nm.

phosphate buffer, pH 7.6 containing 0.02% (w/v) sodium azide and operated at a flow rate of 30 ml/hr.

5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide tube gel electrophoresis was carried out by a method essentially due to Laemmli (1970). For the preparation of small pore gel (12.5% cross-linking), 7.5 ml of 30% acrylamide solution, 0.8% N-N'-methylene bisacrylamide, 0.01 ml of N,N,N',N' tetramethylethylenediamine tetra acetic acid and 0.07 ml of 10% ammonium persulphate were mixed. Sodium dodecyl sulphate (0.1%) was also present in the gel. A drop of water was carefully applied on top of the gel and it was left to polymerize for some time. Stacking gel (2% cross linking) was layered on top of the small pore gel and a drop of water was carefully layered over it as before.

For the preparation of sample, the protein was dialyzed against 0.025M Tris, 0.192 M glycine buffer, pH 8.3 containing 0.1% SDS. Then the sample was heated for 30 minutes in a boiling water bath. After cooling the sample, few drops of glycerol containing 0.1% (w/v) bromophenol blue were added. The sample was reduced by adding 0.1M 2-mercaptoethanol. An appropriate volume of this mixture (0.05 - 0.1 ml) containing 60-80 ug of protein was then applied to the gel tubes. The

electrophoresis was carried out for 6-7 hours in 0.025M Tris, 0.192 M glycine, buffer pH 8.3 using a current of 6 mA per tube. The gels were removed from the gel tubes with the help of a long needle attached to a syringe. These were then stained with 0.2% (w/v) coomassie brilliant blue R-250 dye in 25% (v/v) methanol and 10% (v/v) acetic acid and destained mechanically with 10% (v/v) acetic acid. The relative mobility (Rm) of the proteins were calculated by the standard procedure.

6. Determination of inhibitory activity

The inhibitory activity of alpha-1-PI was measured against trypsin. The substrate used for trypsin was BAPNA. The enzyme induced hydrolysis of BAPNA produced p- nitroanilide(pNA) which absorbed maximally at 410 nm. The absorbance at 410 nm was taken to represent the concentration of hydrolyzed product. A fixed concentration of enzyme was incubated with different concentrations of alpha-1-PI in 0.1 M Tris-HCl buffer, pH 8.0 containing 0.01 M $CaCl_2$ for 30 minutes at 37°C. The residual enzyme activity for trypsin catalyzed hydrolysis of BAPNA was measured as described by Erlanger, et al., (1961). The final concentration of BAPNA taken for the reaction was 0.8 mM.

7. Isolation and purification of alpha-1-PI

Alpha-1-PI was isolated from discarded human plasma by modifying the method described by Musiani and

Tomasi (1976). The serum was precipitated with an equal volume of saturated ammonium sulphate. The precipitate thus obtained was discarded and the supernatant was further subjected to 80% ammonium sulphate concentration. The 80% $(NH_4)_2SO_4$ precipitate thus obtained by centrifugation was dialyzed extensively against 10mM sodium phosphate buffer, pH 7.6. The crude inhibitor was further purified by ion exchange chromatography on DEAE-cellulose column (2.5 X 5.8 cm) equilibrated with 10mM sodium phosphate buffer, pH 7.6. The protein was eluted batchwise with 10mM sodium phosphate buffer, pH 7.6 containing 0.05 M, 0.1 M, 0.15 M and 0.2 M NaCl. The fractions containing alpha-1-PI located using BAPNA as substrate for trypsin were pooled and concentrated by $(NH_4)_2SO_4$ precipitation. The inhibitor was further purified by extensively dialyzing the precipitate against 10 mM Tris-Hcl buffer pH 7.6, containing 0.15 M NaCl, 1mM $CaCl_2$, $MnCl_2$ and $MgSO_4$ and applying on concanvalin A bound Sepharose 4B column (2.2 X 5.3 cm). The unbound material was eluted with 10 mM Tris-HCl buffer, pH 7.6 containing 0.15M NaCl, 1 mM $CaCl_2$, $MnCl_2$ and $MgSO_4$ and alpha-1-PI was subsequently eluted with 0.5 M glucose in the same buffer. The fractions showing antitryptic activity were pooled, concentrated and chromatographed on Sephadex G-100 column (2.4 X 79 cm) in 10mM sodium phosphate buffer, pH 7.6. The alpha-1-PI fractions were pooled and concentrated.

Results and Discussion

RESULTS AND DISCUSSION

A. Isolation, purification and molecular weight of alpha-1-PI

Alpha-1-proteinase inhibitor was isolated from human plasma by salt fractionation followed by ion exchange chromatography and chromatography on Con A Sepharose 4B. The results are summarised in Table VI. Starting from 500 ml of plasma containing 30.965 gm protein, 2.310 gm crude inhibitor was isolated by ammonium sulphate fractionation. It was purified by chromatography on DEAE-cellulose column equilibrated with 10 mM sodium phosphate buffer, pH 7.6. The protein was eluted batchwise with sodium phosphate buffer containing increasing NaCl (0.05-0.2M). Four peaks were obtained out of which fractions under peak C showed antitryptic activity using BAPNA as substrate (see Fig.8). Ion exchange chromatography resulted in significant purification of protein. However the purified inhibitor contained significant amount of impurity. Consequently, when protein was passed on Con A Sepharose 4B column equilibrated with metallized Tris buffer, pH 7.6, the inhibitor being glycoprotein was specifically bound to column. Elution with operating buffer gave a single wash through peak and about 160 mg of protein was specifically eluted with 0.5 M glucose. The activity was located only in fractions specifically

TABLE VI

PROTEIN YIELD AT VARIOUS STAGES OF PURIFICATION OF HUMAN ALPHA-1-PROTEINASE INHIBITOR

Step	Total protein (mg)	Total specific activity*	Fold purification	Percent recovery
Plasma	30965	500	1	--
50-80% $(NH_4)_2$ SO_4 fraction	2130	75.6	2.2	6.9%
Ion exchange chromatography on DEAE cellulose	1129	70	3.8	3.6%
Affinity chromatography on Con A Sepharose 4B	160	58	22.3	0.5%
Gel filtration on Sephadex G-100	125	53	26.3	0.4%

*1 specific unit is defined as amount of inhibitor required for complete inhibition of 1 mg of trypsin.

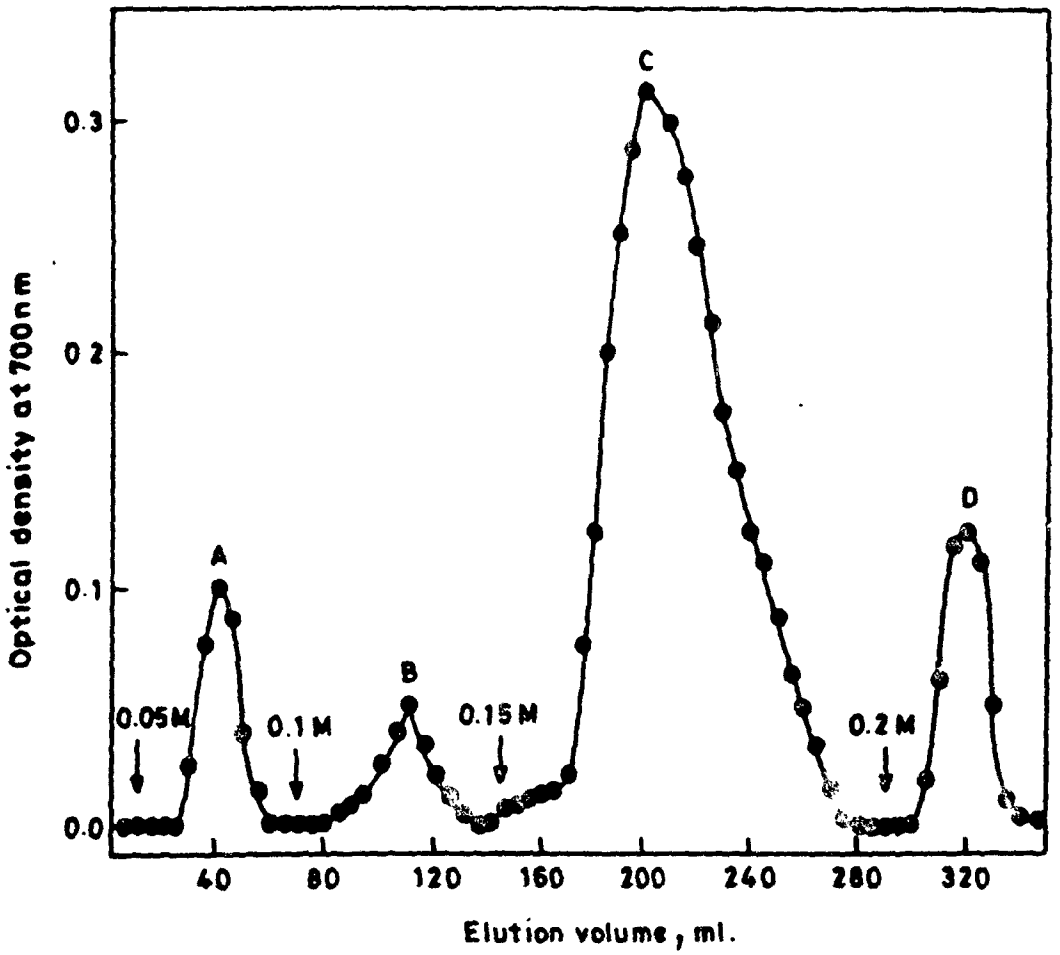


Fig. 8: Ion exchange chromatography of salt fractionated crude human alpha-1-proteinase inhibitor on DEAE-cellulose column.

About 85 mg of protein in 5 ml of 10 mM sodium phosphate buffer, pH 7.6 was applied to DEAE-cellulose column (2.5 x 5.8 cm) equilibrated with operating buffer. The protein was eluted batchwise with 0.05M, 0.1M, 0.15M and 0.2M and 0.2M NaCl in the buffer in 5 ml fractions at flow rate of 20 ml/hr. The protein concentration was determined by the method of Lowry, et al., (1951). The antitryptic activity was located in peak C.

eluted with 0.5 M glucose. Protein fractions under peak B of Fig. 9 were pooled and gel filtered on Sephadex G-100 column equilibrated with 10mM sodium phosphate buffer, pH 7.6, a single protein peak was obtained. This suggested the size homogeneity of alpha-1-PI. Finally, 125 mg of size homogeneous alpha-1-PI was obtained from 500 ml of human plasma.

SDS-PAGE of the purified alpha-1-PI in 12.5% cross-linked gel at pH 8.3 yielded a single coomassie stainable protein band under reducing and non reducing conditions (see Fig.10). The marker proteins, namely bovine serum albumin, ovalbumin, chymotrypsinogen A, cytochrome c were electrophoresed under identical conditions. The relative mobilities of marker proteins and alpha-1-PI are listed in Table VII. A plot of log M versus Rm was obtained by method of least squares and is given in Fig.11; the curve fits the equation:

$$\text{Log } M = 5.182 - 1.204\ Rm \qquad (2)$$

Corresponding to Rm of 0.36, the molecular weight of alpha-1-PI was computed with the help of equation (2) to be 56,000. The latter is only 9.8% higher than the literature value of 51,000.

The elution profile of the inhibitor on Sephadex G-100 is shown in Fig.12. Marker proteins namely bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome c were passed through the column under identical conditions and the results are graphically

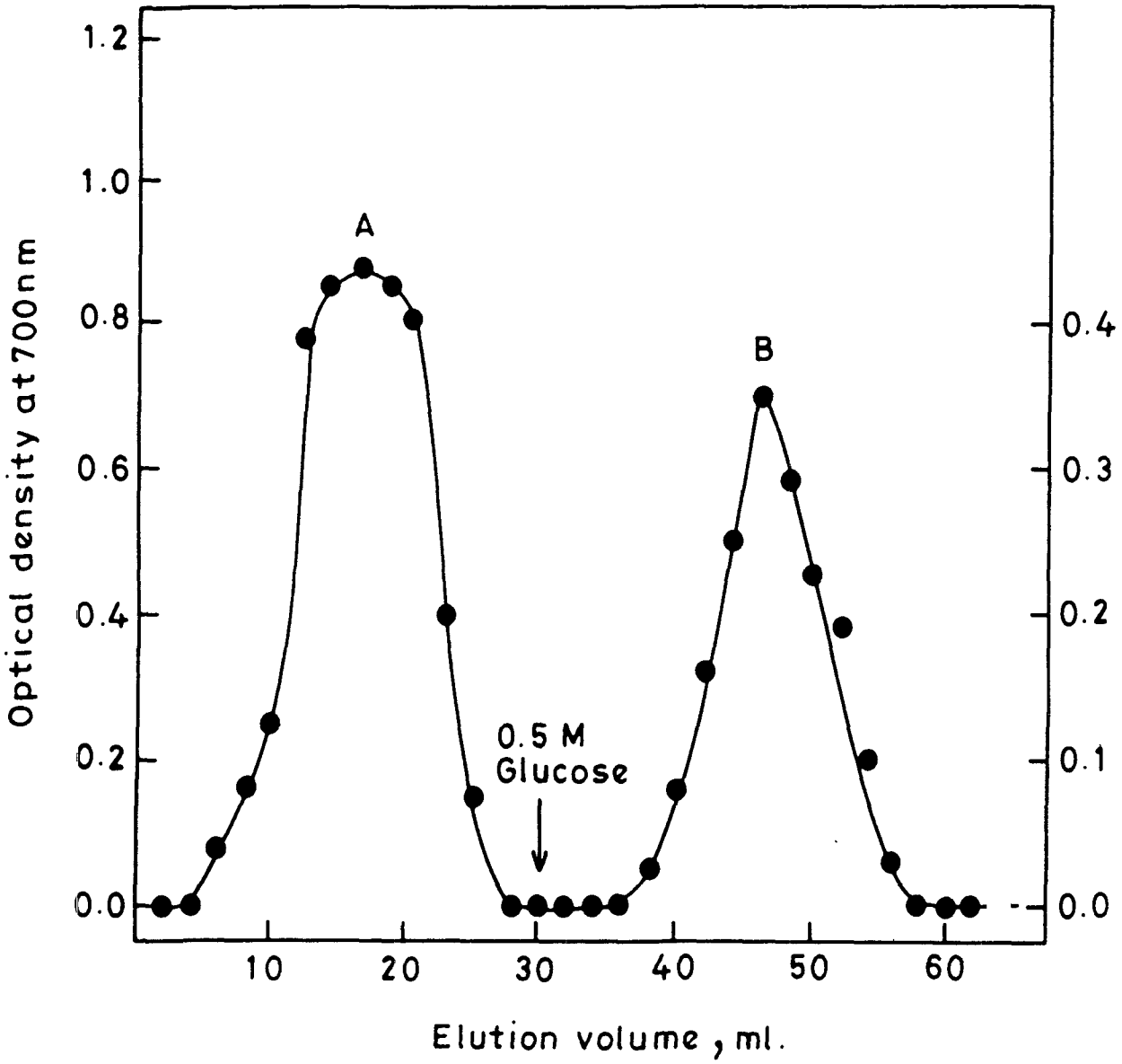


Fig. 9: Chromatography of human alpha-1-proteinase inhibitor on Con A Sepharose 4B column.

About 70 mg of protein in 5 ml of 10 mM metallised Tris-HCl buffer, pH 7.6 was applied on the column (2.2 x 5.3 cm) and eluted with the buffer alone at a flow rate of 5 ml/hr. The protein fractions in wash through peak A was devoid of antitryptic activity. The protein fractions under peak B were obtained by specific elution of glycoprotein with 0.5 M glucose in operating buffer at a flow rate of 30 ml/hr. The column was monitored by the method of Lowry, et al., (1951). The antitryptic activity was locoated in fractions under peak B.

Fig. 10: Sodium dodecyl sulphate polyacrylamide gel electrophonetic pattern of human alpha-1-proteinase inhibitor.

About 60 ug of inhibitor was electrophoresed in 0.025 M Tris 0.192 M glycine buffer, pH 8.3 containing 0.1% SDS in 12.5% polyacrylamide gel for 6 hours at 6 mA current flow per tube. The gel was stained with 0.2% coomassic brilliant blue R-250 and destained mechanically with 10% (V/V) acetic acid as described in the 'Experimental Section'.

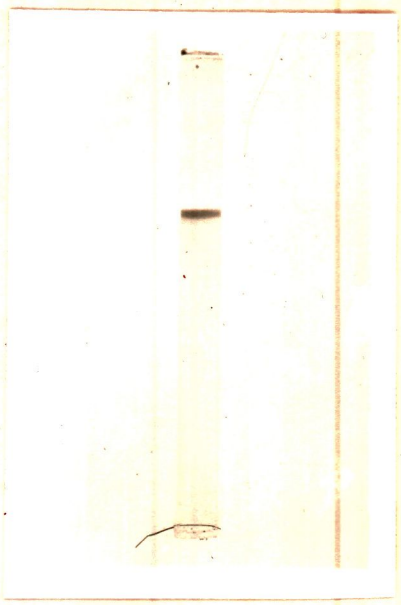


TABLE VII

MOLECULAR WEIGHTS AND RELATIVE MOBILITIES OF MARKER PROTEINS USED IN SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

S.No.	Protein	Molecular weight[a]	Log M.	Rm
1.	Bovine serum albumin	68,000	4.83	0.30
2.	Ovalbumin	43,000	4.63	0.44
3.	Chymotrypsinogen A	25,700	4.44	0.63
4.	Cytochrome c	11,700	4.07	0.92
5.	Alpha-1-PI	--	--	0.36

a. Weber and Osborn (1969).

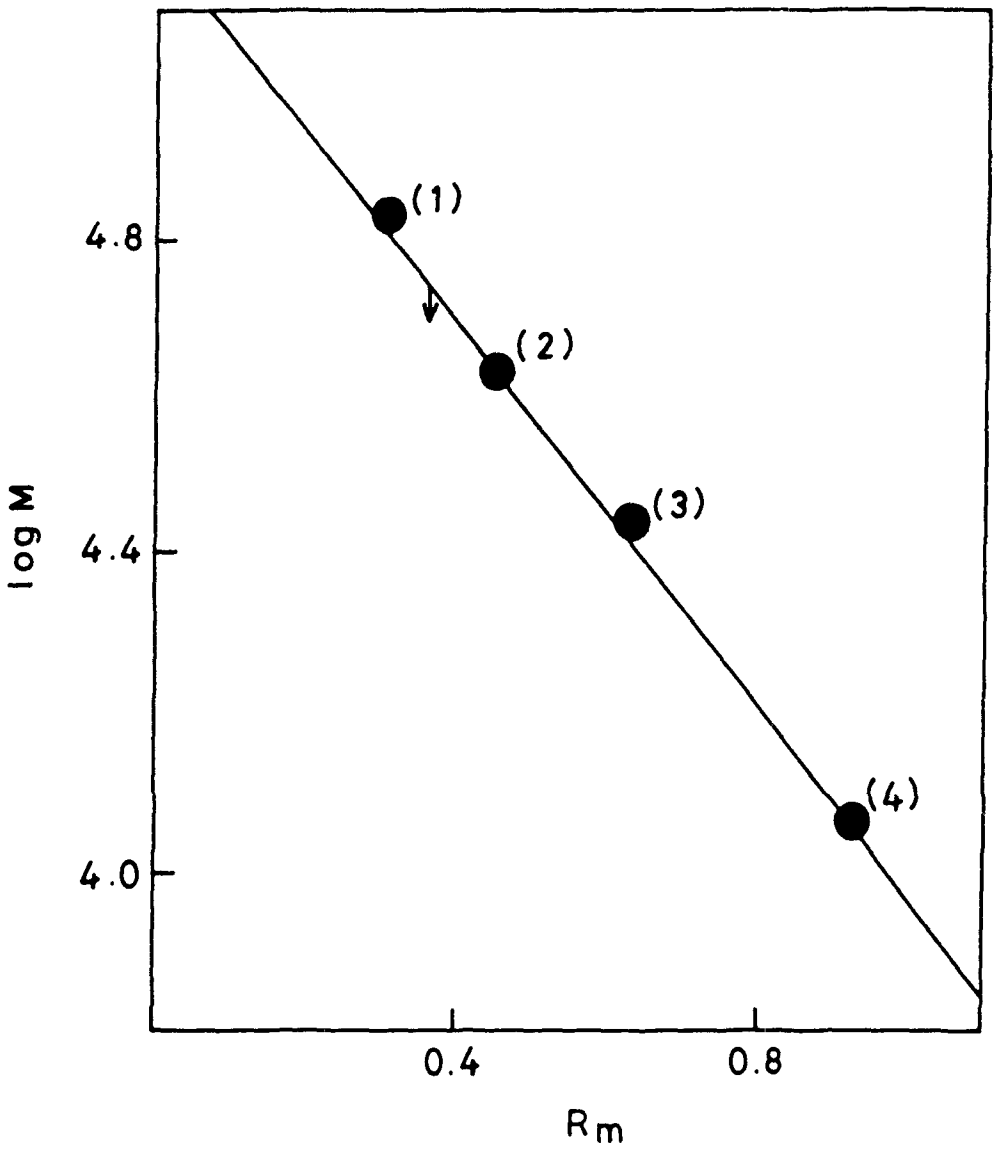


Fig. 11: Plot of Rm values of marker protein versus logarithm of molecular weight.

The marker proteins were (1) bovine serum albumin (2) ovalbumin (3) chymotrypsinogen A (4) cytochrome c. The Rm value of alpha-1-PI is indicated by an arrow. The straight line obtained by the method of least squares fits the equation:

Log M = 5.182 - 1.204 Rm

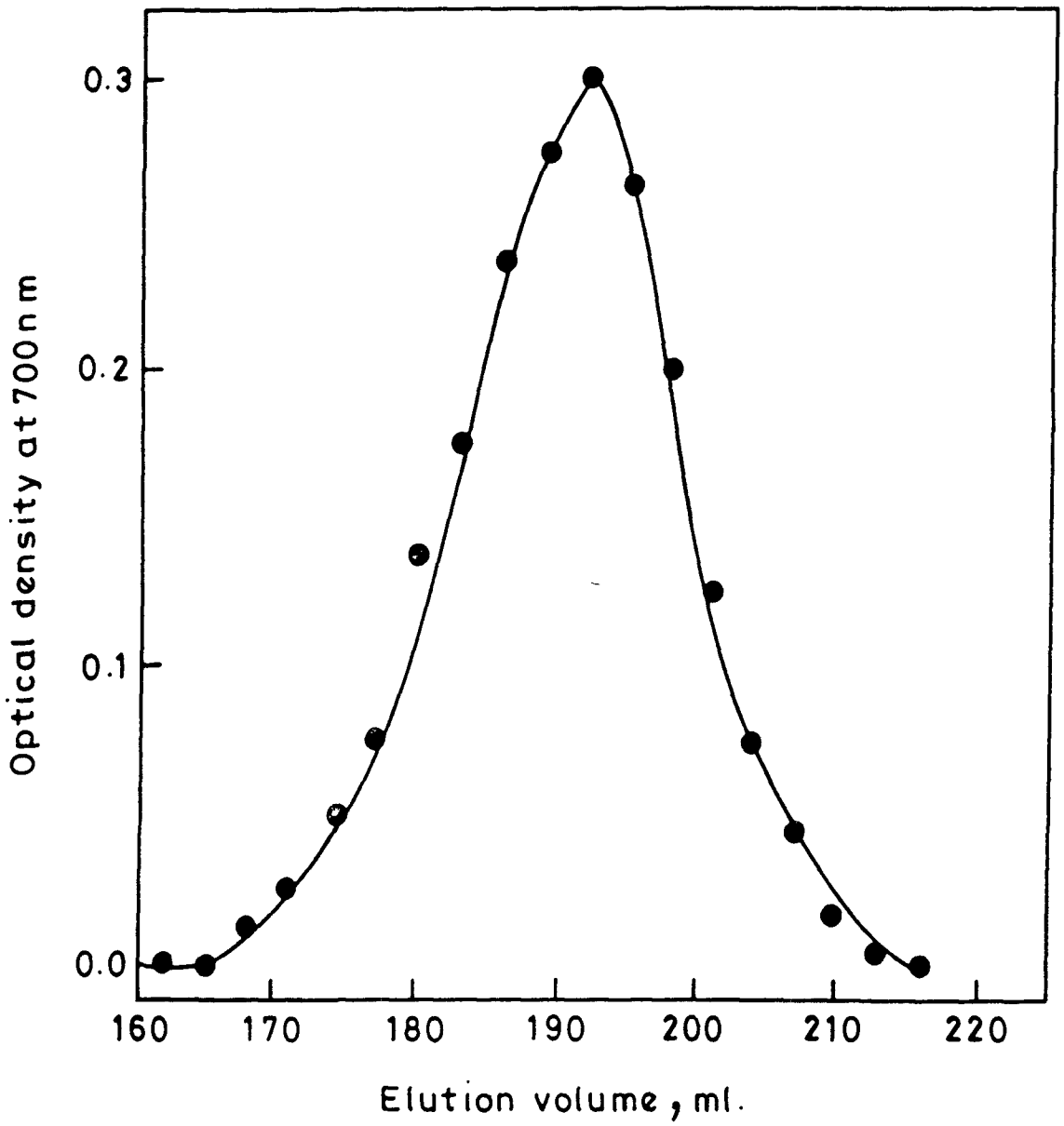


Fig. 12: Gel chromatography of purified human alpha-1-proteinase inhibitor on Sephadex G-100 column.

About 15 mg of inhibitor was applied to the column (2.4 x 79 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.6. The protein was eluted in 3 ml fractions at a flow rate of 20 ml/hr. The protein was monitored by the method of Lowry, et al., (1951).

shown in Figs. 13, 14, 15, 16, 17. The elution volume of Blue Dextran from the column was determined to be 135ml (see Fig.7) The Ve/Vo of marker proteins as well as alpha-1-PI are summarised in Table VIII. Analysis of the results by least square method yielded a straight line (see Fig.18) which fits the equation:

$$Ve/Vo = -0.589(\log M) + 4.239 \qquad (3)$$

The ratio of Ve/Vo for alpha-1-PI was found to be 1.42 which according to equation (3) corresponds to molecular weight of 60,000, which is significantly higher than the reported value of 51,000. This discrepancy is understandable in view of the fact that alpha-1-PI is a glycoprotein containing 13% carbohydrate. Since elution volume of a protein in gel chromatography correlates better with its Stokes radius (Ackers, 1970), it is expected to be higher for proteins showing marked degree of hydration. Glycoproteins usually bind more water than non glycoproteins, this generally causes an over-estimation of the molecular weight of glycoproteins by gel filtration. However, the molecular weight values determined by gel filtration and SDS-PAGE are comparable, not identical. These results, taken together, suggest that alpha-1-PI consists of a single polypeptide.

B. Alkaline inactivation of alpha-1-PI

The purified alpha-1-PI was sensitive to pH at extreme pH values. Below pH 5.0, the inhibitor was less

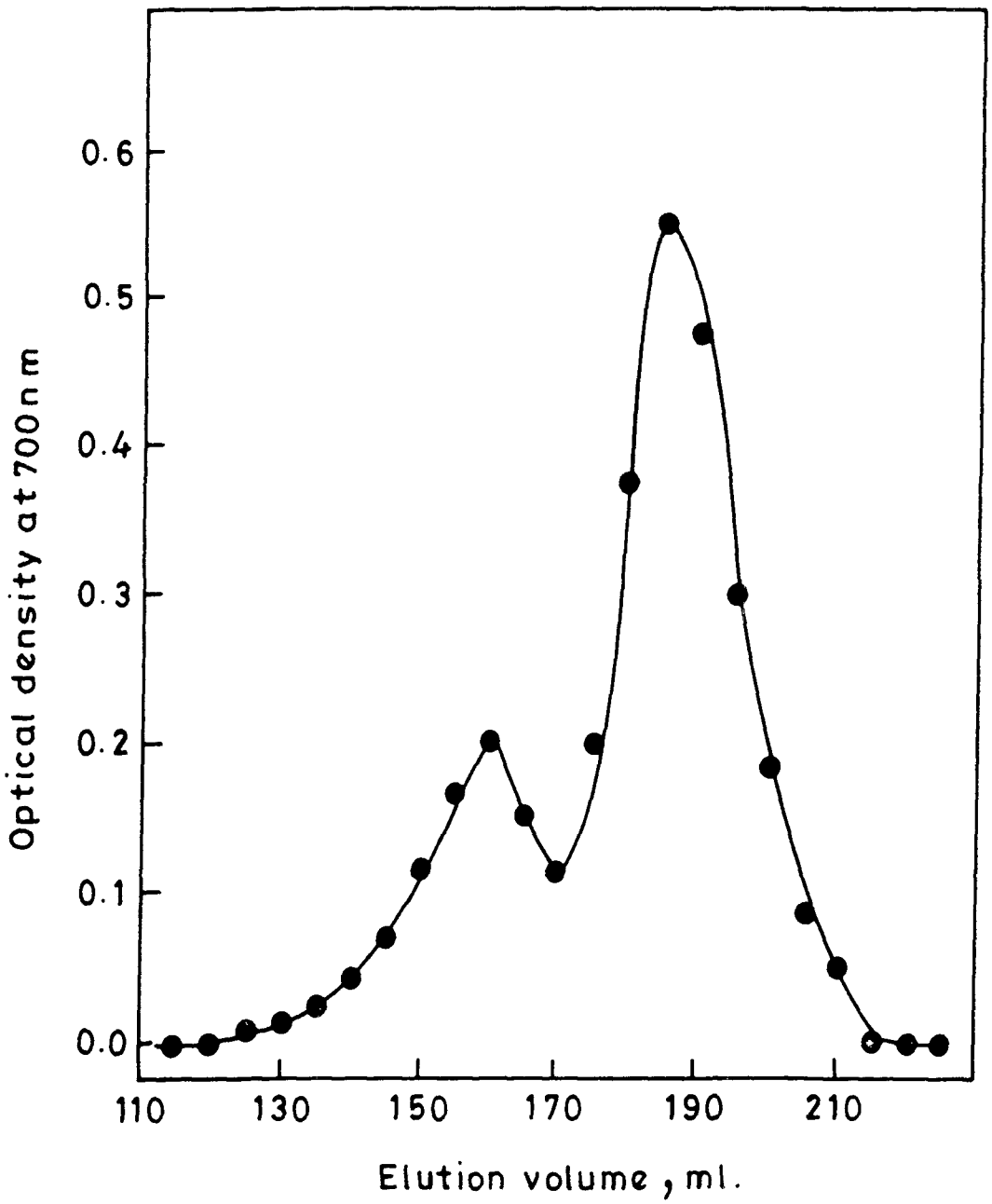


Fig. 13: Gel chromatography of bovine serum albumin on Sephadex G-100 column.

About 10 mg protein was applied to the column (2.4 x 79 cm) equilibrated with 10mM sodium phosphate buffer, pH 7.6. The protein was eluted in 5 ml fractions at a flow rate of 20 ml/hr. The column was monitored by the method of Lowry, et al., (1951).

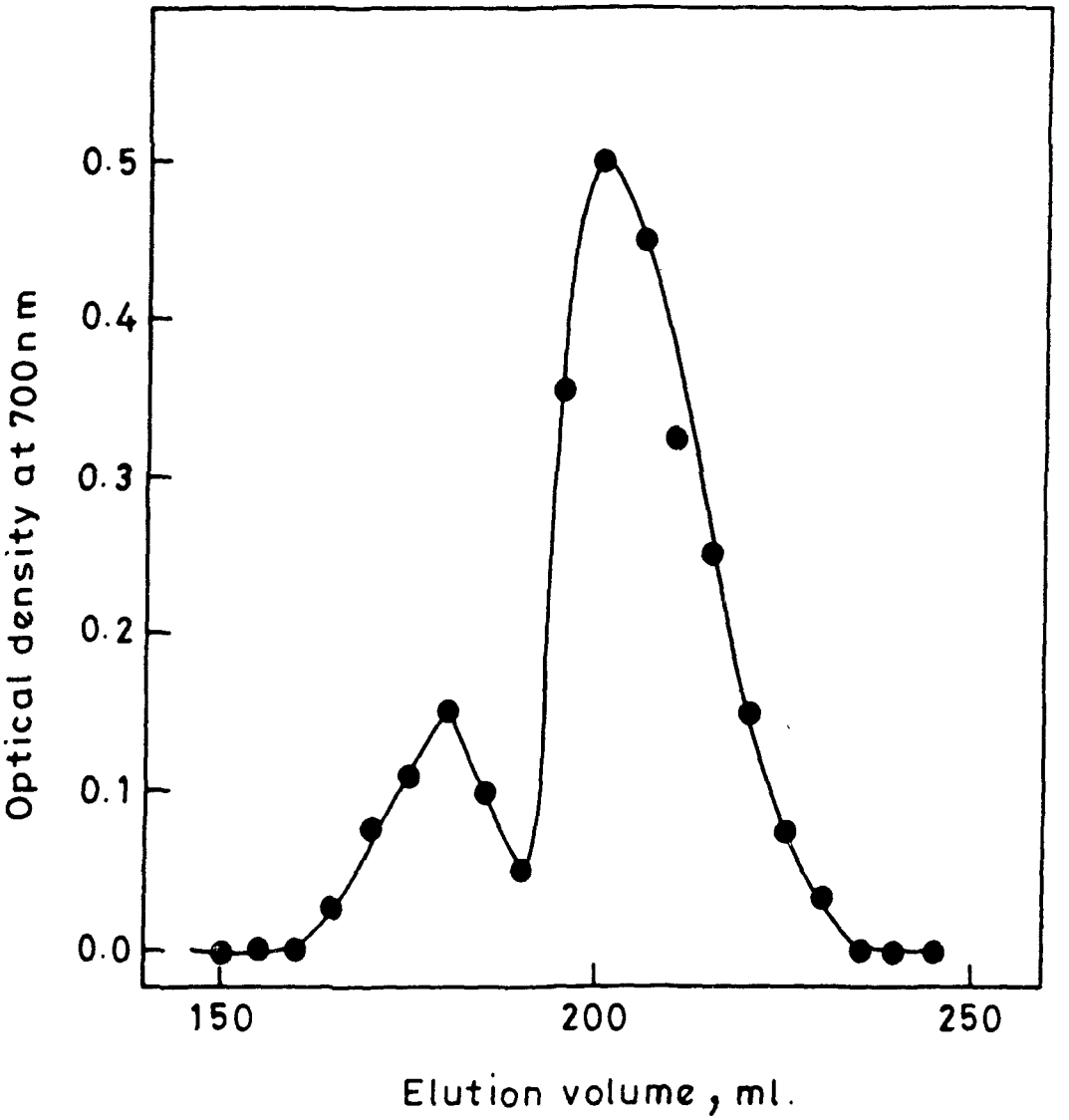


Fig. 14: Gel chromatography of ovalbumin on Sephadex G-100 column.

Experimental conditions were the same as given in the legend to Fig. 13.

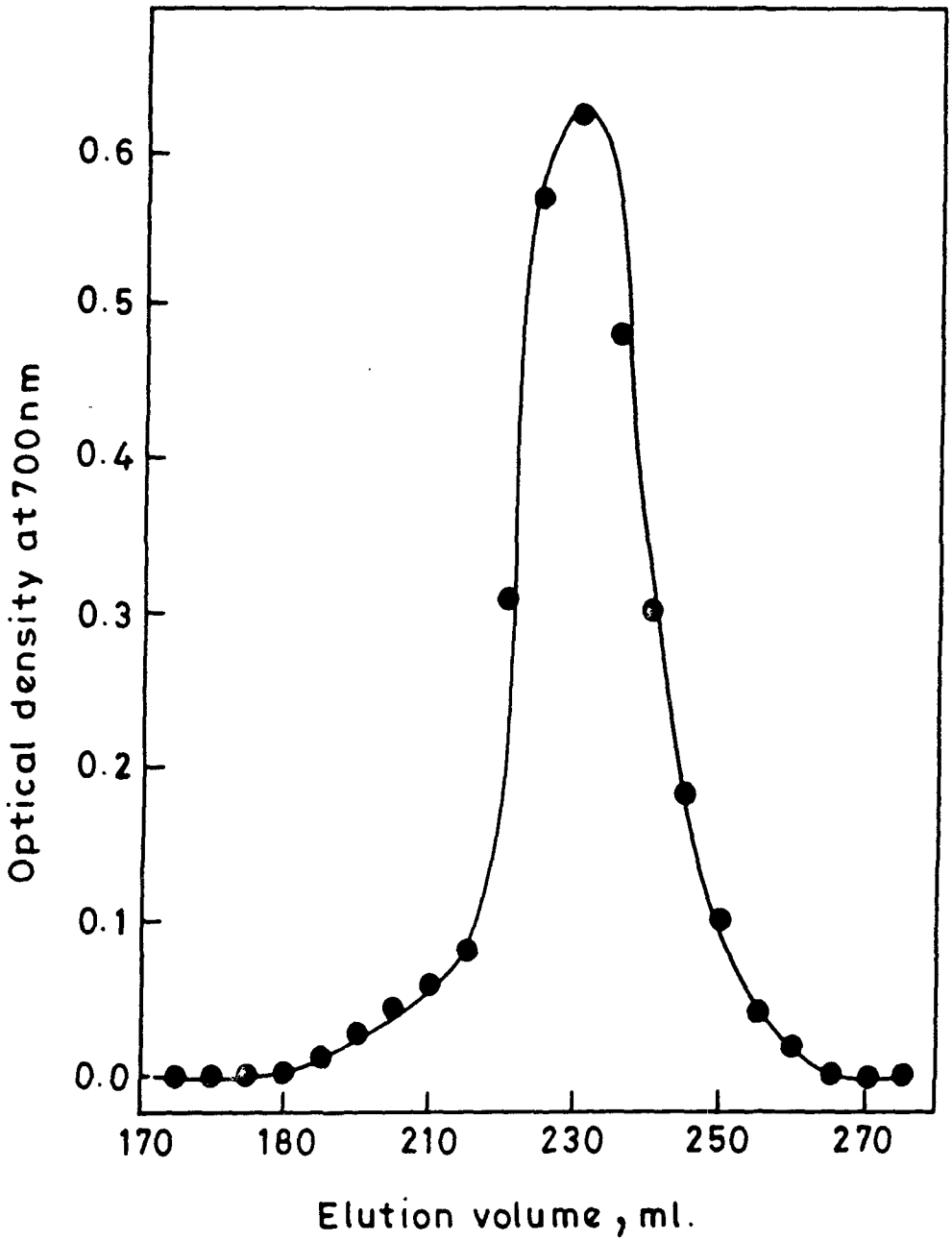


Fig. 15: Gel chromatography of chymotrypsinogen A on Sephadex G-100 column.

Experimental conditions were the same as given in the legend to Fig. 13.

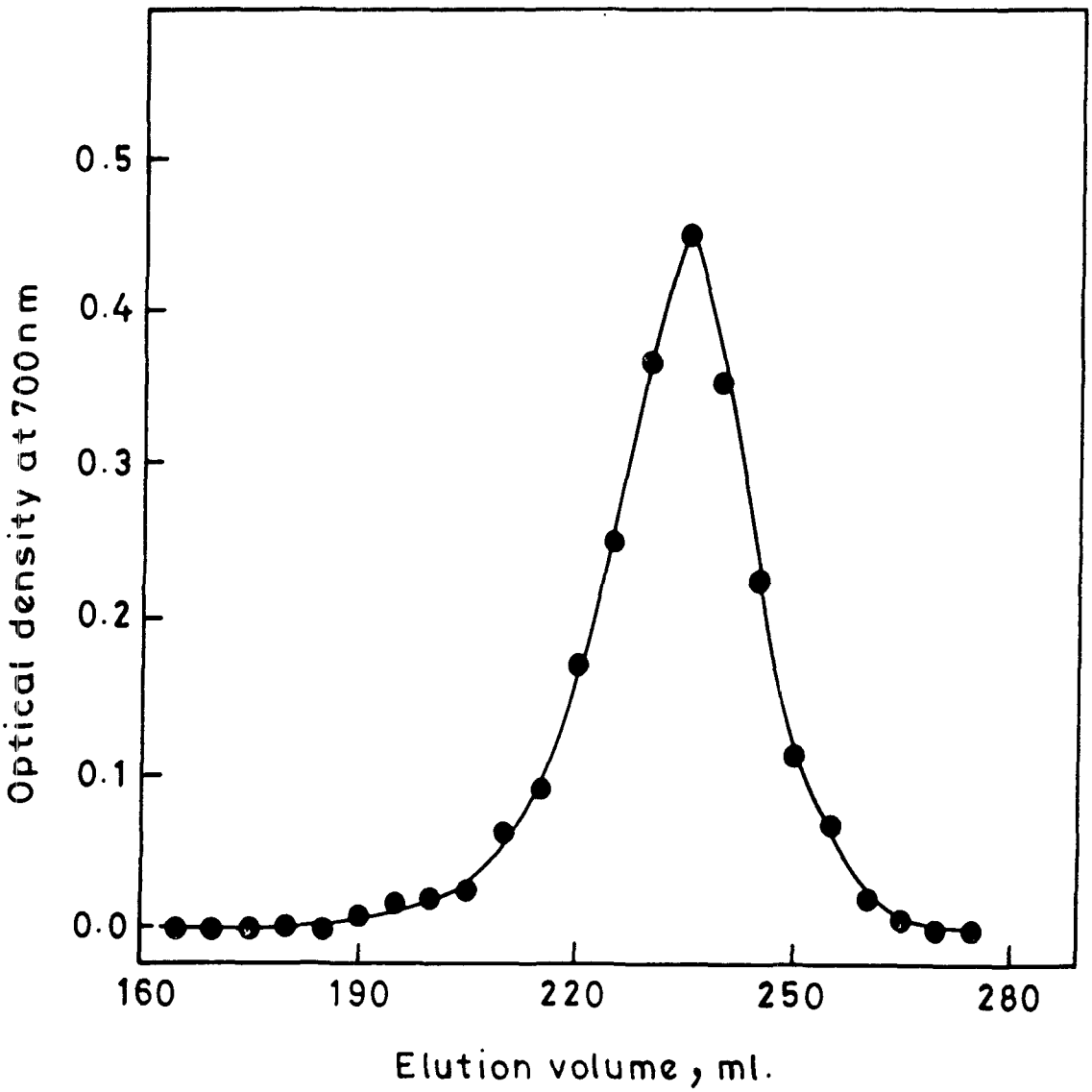


Fig. 16:Gel chromatography of myoglobin on Sephadex G-100 column.

Experimental conditions were the same as given in the legend to Fig. 13.

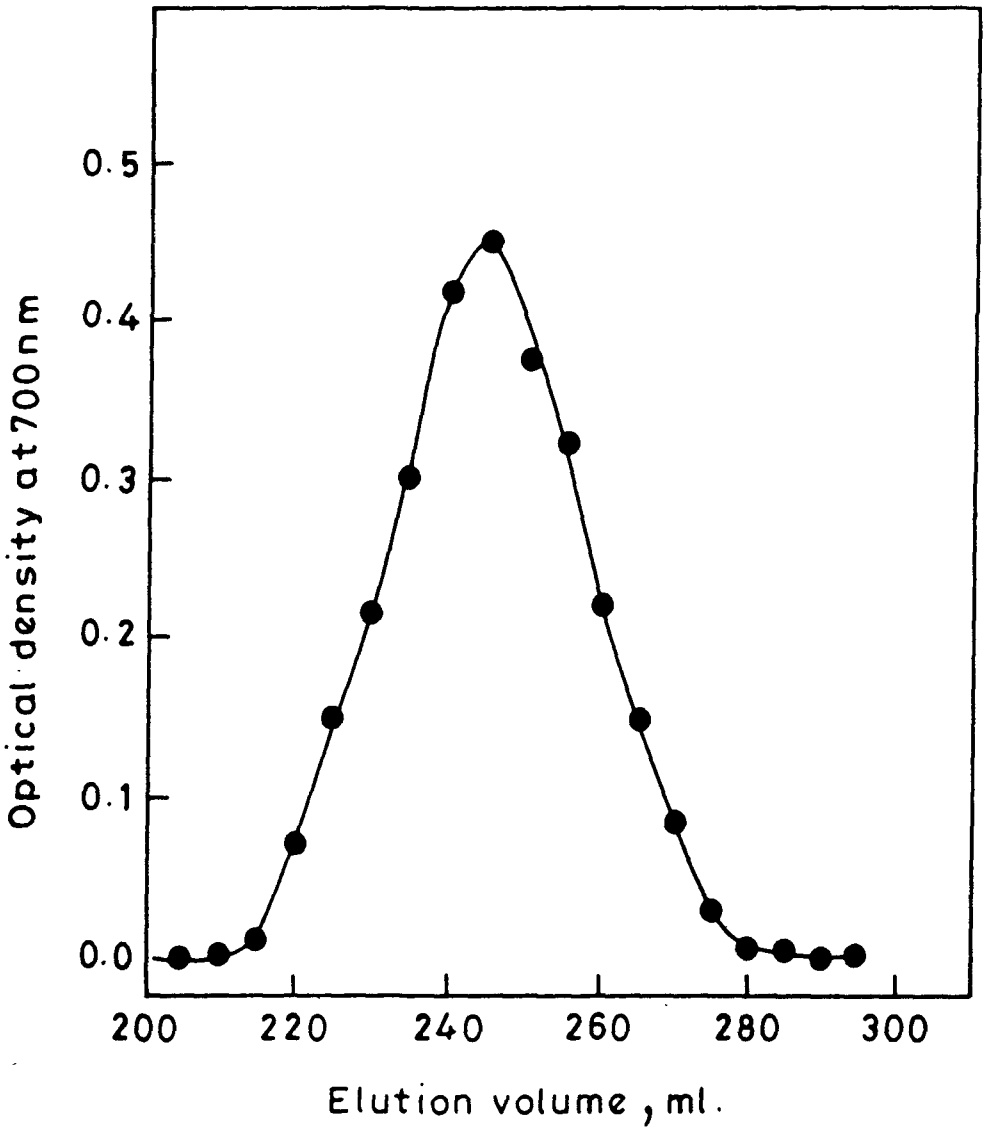


Fig. 17: Gel chromatography of cytochrome c on Sephadex G-100 column.

Experimental conditions were the same as given in the legend to Fig. 13.

TABLE VIII

GEL FILTRATION DATA FOR THE MARKER PROTEINS OBTAINED ON SEPHADEX G-100 COLUMN

S.No.	Protein	Molecular weight[a]	Log M	Ve(ml)	Ve/Vo
1.	Bovine serum albumin	68,000	4.83	185	1.37
2.	Ovalbumin	43,000	4.63	200	1.53
3.	Chymotrypsinogen A	25,700	4.44	230	1.7
4.	Myoglobin	17,200	4.23	235	1.74
5.	Cytochrome c	11,700	4.07	245	1.81
6.	Alpha-1-PI	--	--	192	1.42

a. Weber and Osborn (1969).

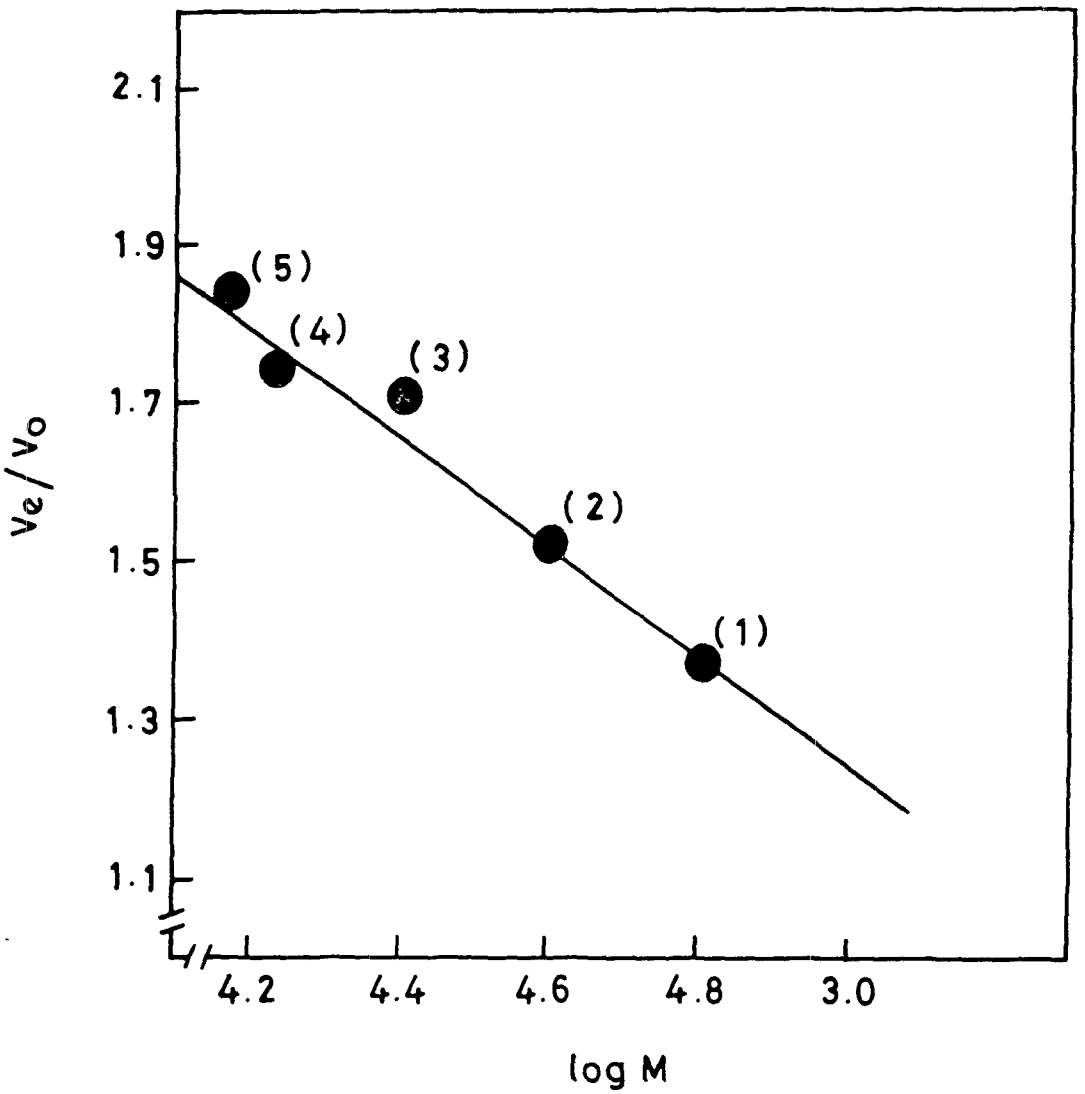


Fig. 18: Plot of V_e/V_o values of marker proteins versus logarithm of molecular weight.

The marker proteins were (1) bovine serum albumin (2) ovalbumin (3) chymotrypsinogen A (4) myoglobin (5) cytochrome c. The V_e/V_o value of alpha-1-PI is indicated by an arrow. The straight line computed by the help of least squares, fits the equation:

$$V_e/V_o = - 0.589 \text{ (Log M)} + 4.239$$

soluble and was completely precipitated from the aqueous solution at pH 2.0. The precipitate dissolved in 8 M urea. However, after replacement of urea with 10mM sodium phosphate buffer pH 7.6, the inhibitory activity could not be restored. The inhibitor showed activity in the pH range 5.0 - 10.0. Above pH 10.0, it underwent inactivation. We report below the results on the time course of inactivation of alpha-1-PI at pH 11.0 at four temperatures namely 25°C, 30°C, 35°C, and 40°C. The buffer used was 10mM sodium carbonate buffer, pH 11.0 containing 0.15M NaCl.

First, alpha-1-PI (1 ml, containing 2.4 mg protein) was mixed with 4 ml of 10 mM sodium carbonate buffer, pH 11.0 and incubated at a given temperature for various lengths of time. Aliquots of 0.1ml of the inhibitor solution were taken and their pH was adjused to 7.6. To this mixture was added 0.1ml of trypsin (containing 0.1 mg protein) in 10mM sodium phosphate buffer, pH 7.6 and the residual tryptic activity was measured against BAPNA. The percent inhibition of tryptic activity, was measured as described in the 'Experimental Section'. A plot of percent inhibition versus time in hours was obtained at four different temperatures. The results are graphically shown in Fig.19.

The results of Fig.19 were analysed according to first order kinetics law in the form of a plot between

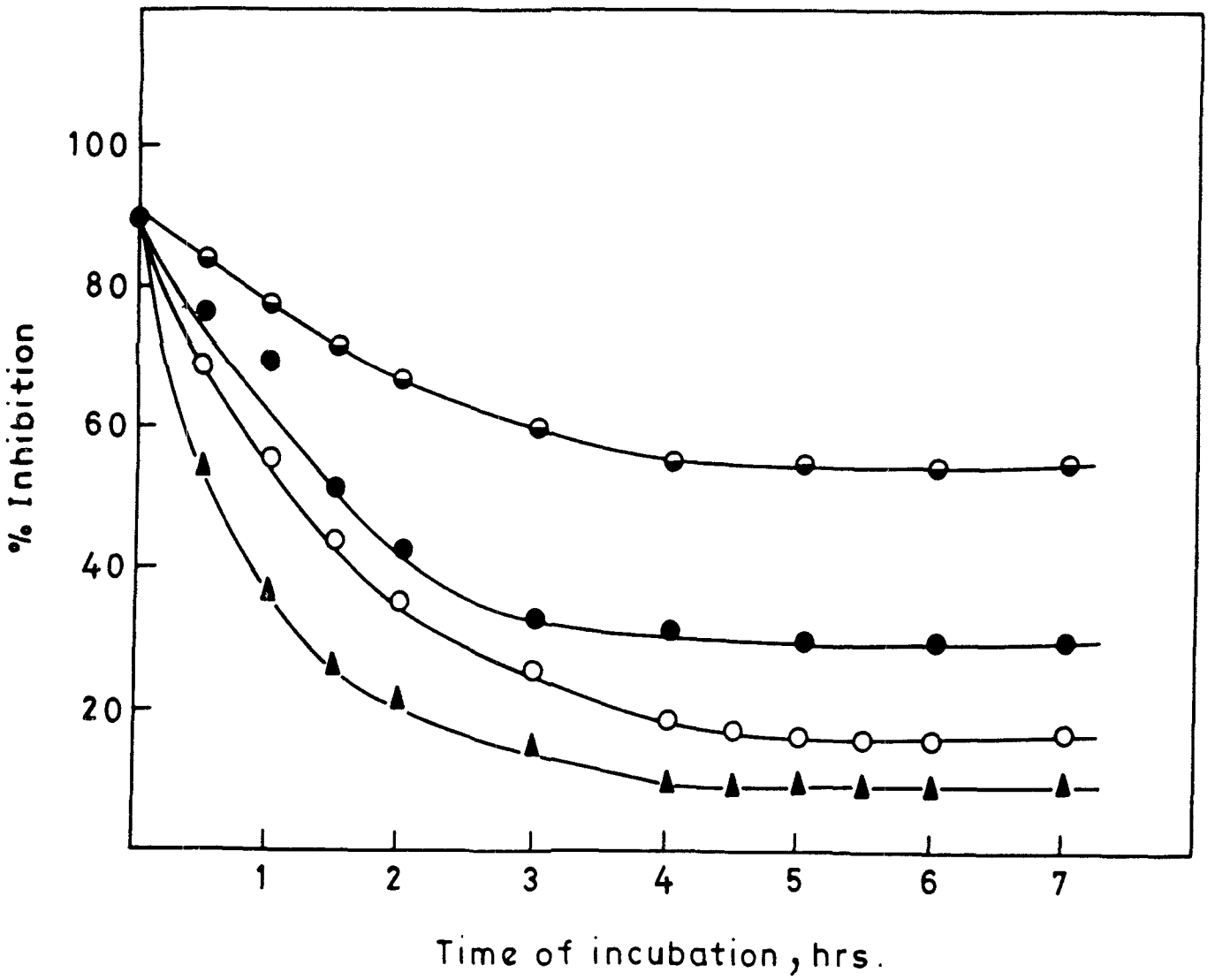


Fig. 19: Time course of the alkaline inactivation of human alpha-1-proteinase inhibitor at different temperatures.

One ml of inhibitor solution containing 2.4 mg of protein was mixed with 4 ml of 10 mM sodium carbonate buffer, pH 11.0, and incubated at 25°C (◐—◐), 30°C (●—●), 35°C (○—○) and 40°C (▲—▲). Aliquots of 0.1 ml were taken at different time intervals and assayed for antitryptic activity as described in the 'Experimental Section'.

log percent inhibition and time. The slope of the straight line at 25°C was found to be 2.28 x 10^{-3} whence the rate constant k, for the inactivation of alpha-1-PI was computed to be 2.28 x 10^{-3}. By similar procedure the rate constants at three other temperatures, viz., 30°C, 35°C, 40°C were computed. The values of k at different temperatures are summarised in Table IX. Arrhenius plot between lnk and 1/T was obtained by the method of least squares. The slope of the straight line shown in Fig.20 was determined to be - 9430.82. The activation energy calculated from the slope comes out to be 18.74 kcal / mole or 78.4 kJ/mole. The value of 18.74 kcal/mole is considerably lower than the activation energy determined for denaturation of proteins. Cathepsin B undergoes alkaline denaturation which is accompanied by large structural changes. (Turk, et al., 1994). The activation energy for the denaturation of Cathepsin B was measured to be 44 kcal/mole. This value is obviously much higher than that found for alkaline inactivation of alpha-1-PI in this study. Our results on circular dichroism spectra of alpha-1-PI in the UV region (200-250 nm) (Fig.21) show that the extent of secondary structure is the same at pH 8.0 and pH 11.0, although the inhibitor exhibits activity at pH 8.0 and is devoid of activity at pH 11.0. Thus, alkaline inactivation of alpha-1-PI does not appear to be accompanied by major conformational

TABLE IX

FIRST ORDER RATE CONSTANT FOR THE ALKALINE INACTIVATION OF ALPHA-1-PROTEINASE INHIBITOR AT DIFFERENT TEMPERATURES

S.No.	Temperature	Rate Constant (min^{-1})
1.	25°C	$2.28x10^{-3}$
2.	30°C	$6.0x10^{-3}$
3.	35°C	$6.65x10^{-3}$
4.	40°C	$12.28x10^{-3}$

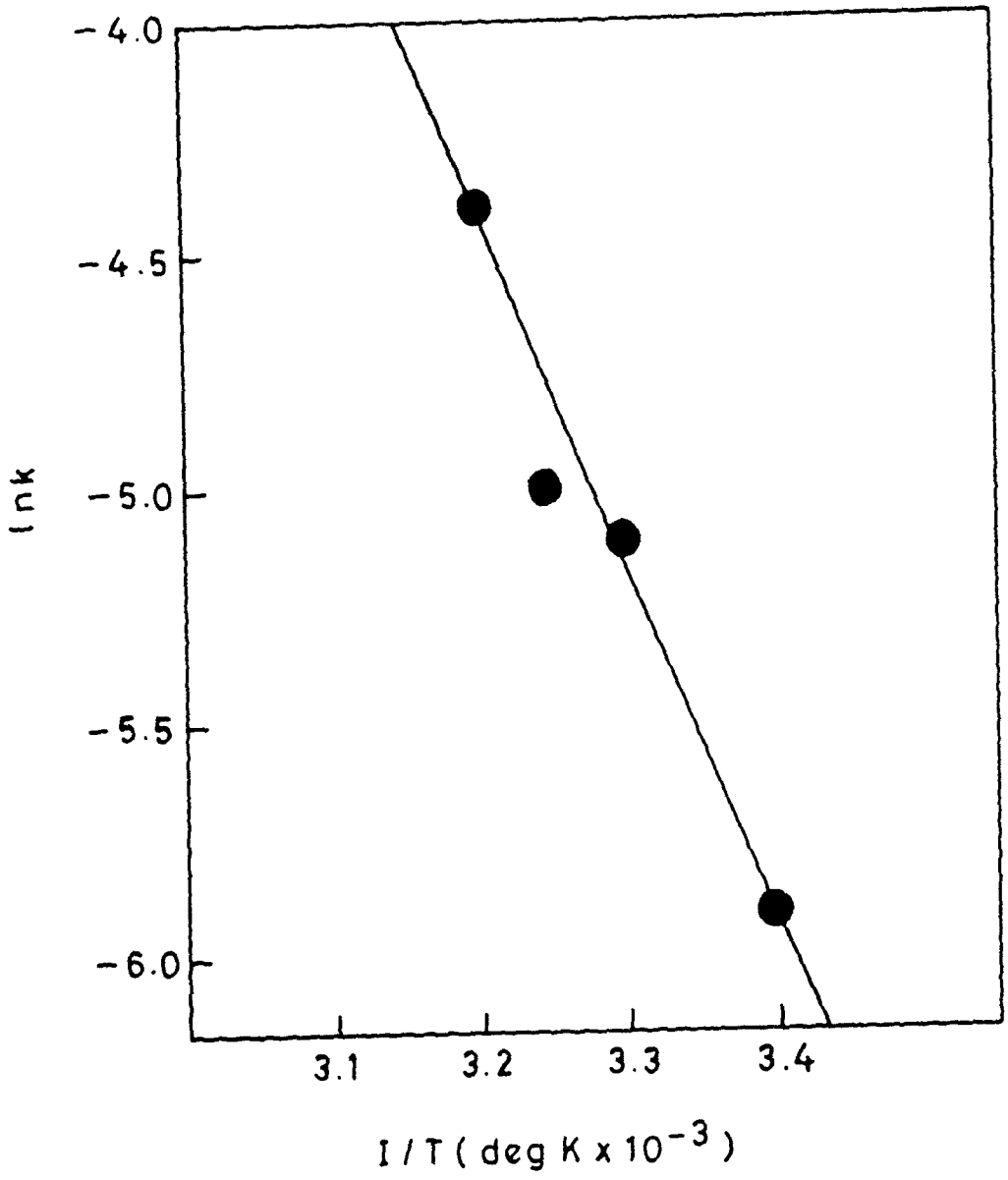


Fig. 20: Arrhenius plot for the alkaline inactivation of human alpha-1-proteinase inhibitor.

A linear plot between ln k versus I/T was obtained by method of least squares. The slope of this line was found to be -9430.82 which yielded a value of 18.74 kcal/mole for activation energy of the alkaline inactivation of alpha-1-PI.

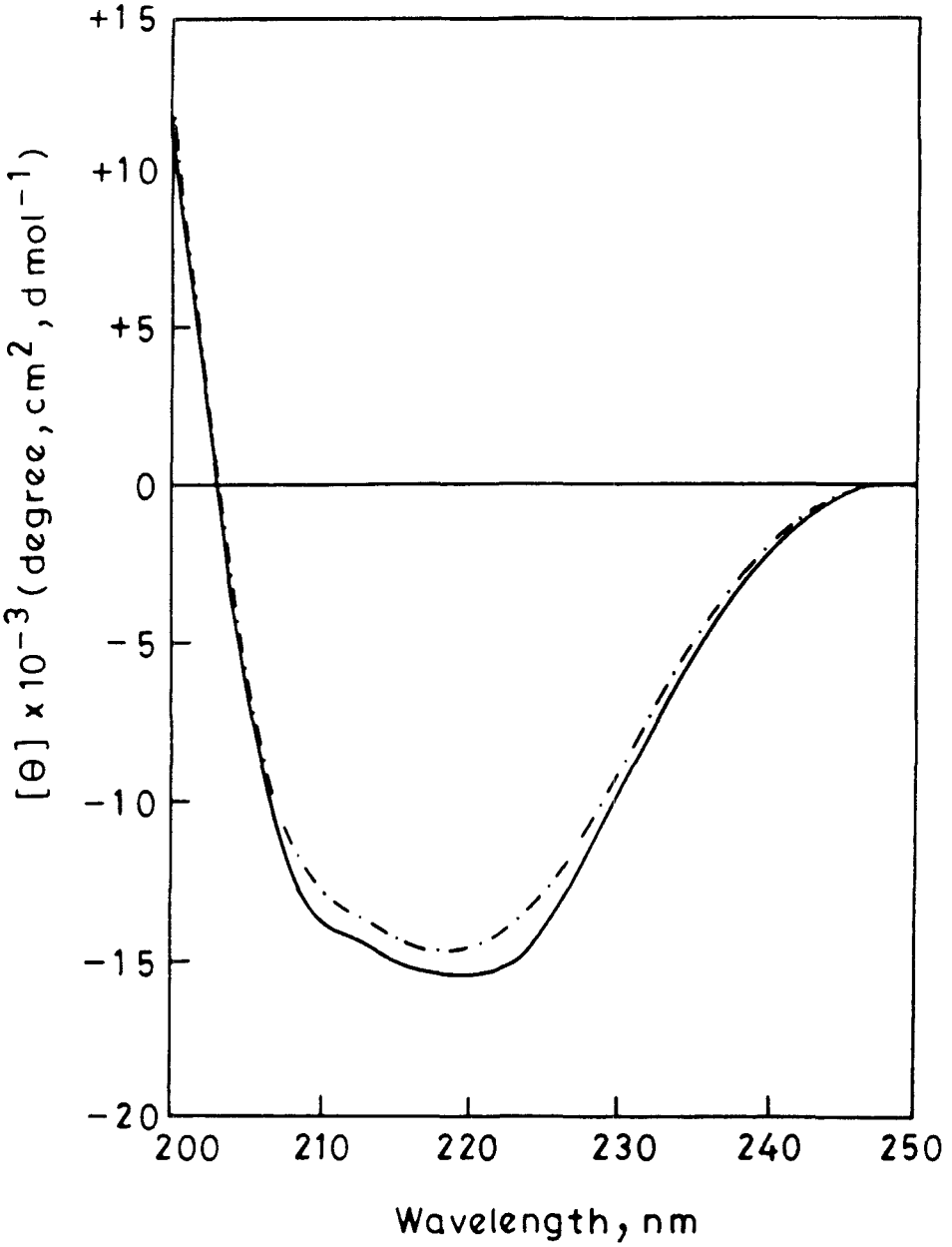


Fig.21 : Circular dichoroism spectra of human alpha-1-proteinase inhibitor.

The far ultraviolet CD spectra of alpha-1-PI (0.22 mg/ml) was carried out at pH 8.0 (⟝⟞) in 10 mM sodium phosphate buffer containing 0.1 M NaCl and at pH 11.0 (·—·—·) in 10 mM sodium carbonate buffer containing 0.1 M NaCl. The spectra was recorded at 20°C.

changes. It is conceivable that exposure of inhibitor to alkaline pH produces small perturbation which does not significantly affect the secondary structure on the whole. Alternatively, the inactivation may be the result of changes in the tertiary conformation of the protein.

Bibliography

BIBLIOGRAPHY

Abrams,W.R., Cohen, A.B., Daimano, V.V., Elirez, A., Kimbel, P., et al., (1981a) J.Clin. Invest. **68**, 1132-1139.

Abrams,W.R., Kimbel, P., and Weinbaum, G., (1978) Biochemistry **17**, 3556-3561.

Abrams, W.R., Weinbaum, G., Weissbauch, I., Weissbauch, H., and Brot, N. (1981b) Proc. Natl. Acad. Sci. U.S.A. **78**, 7483-7486.

Ackers, G.K., (1970) Adv. Protein Chem. **24**, 343-446.

Agostini, A.,Schapira, M., Wachtfogel, Y.T., Colman, R.W., and Carrell, S. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 5190-5193.

Ansari, A.A., Kidwai, S.A., and Salahuddin, A. (1975) J. Biol. Chem. **250**, 1625-1632.

Ansari, A.A., and Salahuddin, A. (1973) Biochem. J. **135**, 705-711.

Babin, D.R., Peanasky, R.J., and Goos, S.M. (1984) Arch. Biochem. Biophys. **232**, 143-161.

Bode, W., Epp, E., Huber, R., Laskowski, M., Jr. and Ardelt, W. (1985) Eur. J. Biochem. **147**, 387-395.

Bode, W., and Huber, R. (1991) Curr. Opin. Struct. Biol. **1**, 45-52.

Bode, W., Papamokos, E., Musil, D., Seemuller, U., and Fritz, H. (1986) EMBO. J. **5**, 813-818.

Bode, W., Papamokos, E. and Musil, D. (1987) Eur. J. Biochem. **166**, 673-692.

Braun, J., Dalhoff,K., Wood,W.G., and Weismann K.J. (1994) Eur. Resp. J. **7**, 127-133.

Brodbeck, R.M., and Brown, J.L. (1994) J. Biol. Chem. **269**, 17252-17256.

Busby, J.F., Yu,S.D., and Gan, J.C. (1977) Arch. Biochem. Biophys. **184**, 267-275.

Campos, F.A., and Richardson, M. (1983) FEBS Lett. **152**, 300-304.

Carrell, R.W., and Evans, D.L. (1992) Curr. Opin. Struct. Biol. **2**, 438-446.

Carrell, R.W., Evans, D.L., and Stein, P.E. (1991) Nature **353**, 576-578.

Carrell, R.W., Jeppson, J.O., Laurell, C.B., Brennan, S.O., and Owen, M.C. (1982) Nature **298**, 329-334.

Carrell, R.W., Owen, M.C., Brennan, S. and Vangham, L. (1979) Biochem. Biophys. Res. Commun. **91**, 1032-1037.

Carrell, R.W., and Owen, M.C. (1985) Nature **317**, 730-732.

Carrell, R.W., and Travis, J. (1985) Trends in Biochem. Sci. **10**, 20-24.

Carrell, R.W., Whisstock, J., and Lomas, D.A.(1994) Am. J. Resp. Crit. Care. Med. **150**, 8171-8175.

Chidwick, K., Whichelow, C.E., and Zhang, Z.U. (1994) Arthiritis. Rheum. **37**, 1723-1726.

Cohen, A.B. (1973) J. Biol. Chem. **248**, 7055-7059.

Cohen, A.B. (1975) Biochim. Biophys. Acta. **391**, 193-200. Curiel, D.T., Chytil, A. Courtney, M., and Crystal, R.G. (1989) J. Biol. Chem. **264**, 10477-10486.

Engh, R.A., Wright, H.T., and Huber, R. (1990) Protein Eng. **3**, 469-477.

Eriksson, S. (1965) Acta. Med. Scand. **177**, 1-85.

Erlanger, B.F., Kokowsky, N., and Cohen, W. (1961) Arch. Biochem. Biophys. **95**, 271-278.

Fakuda, S. (1992) Tokyo. Joshi, Ika. Daigaku. Zasshi. **62**, 17-24.

Feste, A., and Gan, J.C.(1978) J. Biol. Chem. **256**, 6374-6380.

Fretz, C., and Gan, J.C. (1978) Biochim. Biophys. Acta. **537**, 226-237.

Garnier, J., Osguthorpe, D.J., and Robson, B. (1978) J. Mol. Biol. **120**, 97-120.

Gollin, P.A., Kalaria, R.N., Rozemullar, A., and Perry, G. (1992) Neuroreport **3**, 201-203.

Graham, J.S., Pearce, G., Meeryweather, J.,Titani, K., Ericsson, L., and Ryan, C.A. (1985a) J. Biol. Chem. **260**, 6555-6560.

Graham, J.S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L., and Ryan, C.A. (1985b) J. Biol. Chem. **260** 6561-6564.

Gressier, B., Lebegue, S., Gosset, P., Brunet, C., Luyckx, M., Dine, T., and Cazin, M. (1994) Fundam. Clin. Pharmacol. **8**, 518-524.

Heimberger, N., Haupt, H., and Schwick, H. (1971) in Proc. Ist. Int. Res. Conf. Proteinase Inhibitors(H. Fritz. H, Tscheche. Eds) pp. 1-12, Berlin: de Gruyter.

Hejgaard, J., Svendsen, I., and Mundy, J. (1983) Carlsberg Res. Commun. **48**, 95-94.

Holak, T.A., Gondal, D., Otlewski, J., and Wilusz, T. (1989) J. Mol. Biol. **210**, 635-648.

Hood, D.B., Huntington, J.A., and Gettins, P.G.W. (1994) Biochemistry **33**, 8538-8547.

Huang, A.Z., and Laurent, P. (1993) Chem. Res. Toxicol. **321**, 274-278.

Hubbard, S.J., Campell, S.F., andThornton, J.M. (1991) J. Mol. Biol. **220**, 507-530.

Huber, R., and Bode, W. (1978) Acc. Chem. Res. **11**, 114-122.

Huber, R., and Carrell, R.W. (1989) Biuochemistry **28**, 8951-8966.

Janoff, A., and Carp, H. (1977) Am. Rev. Respir. Dis. **116**, 65-72.

Janoff, A., Carp, H., Lee, D.K., and Drew, R.T. (1979) Science **206**, 1313-1314.

Johnson, D.A., and Travis, J. (1975) Protides. Biol.Fluids. Proc. Colloq. **23**, 35.

Johnson, D.A., and Travis, J. (1978) J. Biol. Chem. **253**, 7142-7144.

Johnson, D.A., and Travis, J. (1979) J. Biol. Chem. **254**, 4022-4026.

Joubert, F.J. (1984) Phytochemistry **23**, 1401-1406.

Kurachi, K., Chandra, T., Degen, S.J.F., White, T.T., Marchiano, T.L., Woo, S.L.C., and Davie, E.W. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 6826-6830.

Kwon, K.S., Kim, J., Shin, H.S. and Yu, M.H. (1994) J. Biol. Chem. **269**, 9627-9631.

Laemmli, U.K. (1970) Nature. **227**, 680-685.

Laskowski, M., Jr. (1986) in"Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods" (Mendel, F.Ed) pp. 1-17, Plenum Publishing Corporation.

Laskowski, M., Jr. and Kato, I. (1980) Ann. Rev. Biochem. **49**, 593-625.

Laurell, C.B., and Jeppson, J.O.(1975) in "The Plasma Proteins (Putman, F.W. Ed) 2nd Ed. Vol. 1. pp.229-264, Acad. Press. New York.

Lawrence, D.A., Olson, S.T., Palaniappan, S., and Ginsburg, D. (1994) J. Biol. Chem. **269**, 27657-27662.

Lieberman, J. (1976) J. Chest. **70**, 62-67.

Loebermann, H, Tokuoka, R., Deisenhofer, J., and Huber, R. (1984) J. Mol. Biol. **177**, 531-556.

Lomas, D.A., Elliot, P.R., Chang, W.S.,Wardell, M.R. and Carrell, R.W.(1995) J. Biol. Chem. **270**, 5282-5288.

Lomas, D.A., Evans,D.L.I., Finch,J.T. and Carrell, R.W. (1992) Nature **357**, 605-607.

Lomas, D.A., Evans, D.L.I., Stone, S.R., Chang, W.S.W. and Carroll,R.W. (1993) Biochemistry **31**, 2720-2728.

Long,G.L., Chandra, T., Woo, S.L., Davie, E.W., and Kwachi, K. (1984) Biochemistry **23**, 4828-4837.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. **193**, 265-275.

Mahoney,W.C., Hermodson, M.A., Jones, B., Powers, D.D., Corfman, R.S. and Reeck,G.R. (1984) J. Biol. Chem. **259**, 8412-8416.

Mast, A.C. Enghild, J.J., and Salvesen, G. (1992) Biochemistry **31**, 2720-2728.

Matheson, N.R., Gibson, H.L., Hallewell, R.S., Base, P.J., and Travis, J. (1986) J. Biol. Chem. **261**, 10404-10409.

Matheson, N.R., and Travis, J. (1976) Biochem. J. **159**, 495-502.

Mottonen, J., Strand, A., Symersky, J., Sweet, R.M., Danley, D.E., Geoghegan, K.F., Gerard, R.D. and Goldsmith, E.J. (1992) Nature **355**, 270-273.

Musiani, P., and Tomasi Jr.,T.B. (1976) Biochemistry **15**, 798-804.

Odani, S., Koide, T., Ono, T., and Ohnishi, K. (1983) Biochem. J. **213**, 543-545.

Ohlsson, K., Fryksmark, U., and Tegner, H. (1980) Eur. J. Clin. Invest. **10**, 373-379.

Ottonello, L., Dapino, P., and Dallegri, F., (1993) Respiration **60**, 32-37.

Pannell, R., Johnson, D., and Travis, J. (1974) Biochemistry **13**, 5439-5445.

Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. **269**, 15957-15960.

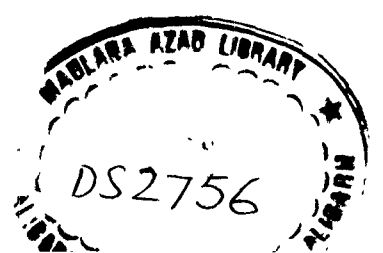
Powell, L.M., and Pain,R.H. (1992) J. Mol. Biol. **224**, 241-252.

Propst, T., Propst, A., Dietze, O., Judmaier, G., Braunsteiner, H., and Vogel, W. (1994) Dig. Dis. **12**, 139-149.

Qasim, M.A., and Salahuddin, A.(1978) Biochim. Biophys. Acta. **536**, 50-63.

Rabin, M., Watson, M., Kidd, V., Woo, S.L.C., Breg, W.R., and Ruddle, F.H. (1986) Somatic. Cell. Mol. Genet. **12**, 209-214.

Read, R.J., and James, M.N.G. (1986) in "Proteinase Inhibitors" (Barrett, A.J., and Salvesen, G.S. Eds) pp. 301-336, Elsevier Science Publishers BV, Amsterdam.



Rosenberg,S., Barr, P.J., Nagarian, R.C., and Hallewell, R.A. (1984) Nature, **312**, 77-80.

Saklatvala, J., Wood, G.C., and White, D.D. (1976) Biochem. J. **157**, 339-351.

Satoh, S., Kurecki, T., Kress, L.F., and Laskowski, M., Sr. (1979) Biochem. Biophys. Res. Commun. **86**, 130-137.

Schulze,A.J., Baumann, U., Knoff,S., Jaeger, E., Huber, R. and Laurell, C. (1990) Eur. J. Biochem. **194**, 51-56.

Schulze, A.J., Huber, R., Degryse, E., Speck, D., and Bischoff, R. (1991) Eur. J. Biochem. **194**, 51-56.

Scott, G.K. (1994) Cell Biol. Int. **18**, 89-93.

Shen, R.F., Li, Y., Sifers, R.N.,Wang, H., Hardick, C., Tsai, S.Y., and Woo, S.L.C. (1987) Nuclic. Acids. Res. **15**, 8399-8414.

Shin, M.S., and Hn, K.J. (1993) Chest. **104**, 1384-1386.

Sires, U.I., Murphy, G., Baragi, V.M., Fliszar, C.J., and Welgus, H.G. (1994) Biochem. Biophys. Res. Commun. **204**, 613-620.

Skriver, K., Wikoff, W.R., Patson, P.A., Tausk, F.,Schapira, M., Kaplan, A.P., and Bock, C.S. (1991) J. Biol. Chem. **266**, 9216-9221.

Stein, P., Leslie, A.J., Finch, J.T., Turnell, W.G., Mclaughlin, P.J. and Carell, R.W. (1990) Nature **347**, 99-102.

Takahara, H., and Sinohara, H. (1982) J. Biol. Chem. **257**, 2438-2446.

Takahara, H., and Sinohara, H. (1983) J. Biol. Chem. **93**, 1411-1419.

Takahashi, H., Nukiwa, T., Satoh, K., Ogushi, F., Brantly, M., Fells, G., Steir, L., Courtney, M., and Crystal, R.G. (1988) J. Biol. Chem. **263**, 15528-15534.

Travis, J., Matheson, M.R., George, P.M., and Carrell, R.W. (1986) Biol. Chem. Hoppe-Seyler. **367**, 853-859.

Travis, J., Owens, M.C., George, P.M., Carrell, R.W., Rosenberg, S., Hallewell, R.A. and Barr, P.J. (1985) J. Biol. Chem. **260**, 4384-4389.

Travis, J., and Salvesen, G.S. (1983) Ann. Rev. Biochem. **52**, 655-709.

Turk, B., Iztok, D., Zeiovnik, E., Turk, D., Gubensek, F., and Turk, V. (1994) Biochemistry **33**, 14800-14896.

Walsh, K.A. (1970) in "Methods in Enzymology" (Perlman, G.E., and Lorand, L. Eds) Vol. 19, pp. 64-108, Academic Press. Inc.

Weber, K., and Osborn, M. (1969) J. Biol. Chem. **244**, 4406-4412.

Wieczorek, M., Otlewski, J., Cook, J., Parks, K., Leluk, K., Wilmowska-Pelc, A., Polanowski, A., Wilusz, T., and Laskowski, M. Jr. (1985) Biochem. Biophys. Res. Commun. **126**, 646-652.

Wright, H.T., (1985) J. Biol. Chem. **259**, 14335-14336.

Wright, T., Qian, H.J., and Huber, R. (1990) J. Mol. Biol. **213**, 513-528.

Zhang, Z., Farrell, A.T., Blake, D.R., Childwick, K., and Winyard, P.G. (1993) FEBS. Lett. **321**, 274-278.

Zhang, Z., Winyard, P.G., Chidwick, K., Murphy, G.,Wardell, M., Carrell, R.W., and Blake, D.R.(1994) Biochim. Biophys. Acta. **1199**, 224-228.

Zhu, X.J., and Chan, S.K. (1987) Biochemistry **246**, 19-23.

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